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SHORT COMMUNICATION

Response of Chick B Islets to Insulin Secretagogues is Comparable to those of Human Islet Equivalents

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ABSTRACT

Context The B islets isolated from 3-5 day old chick respond well to glucose challenge in a similar fashion to those isolated from mouse pancreas. **Objective** To compare insulin secretory response of chick B islets with that of human Islet Equivalents (hIEqs) generated from stem cells. **Methods** Human Umbilical Cord Mesenchymal Stem Cells (UC-MSCs) were differentiated into hIEqs employing three step sequential serum free protocols. **Results** Immunofluorescence staining demonstrated Insulin, C peptide and Glut 2 positivity of both these islets. Static insulin stimulation of these islets in response to glucose, metformin and Gama Amino Butyric Acid (GABA) resulted in increased insulin secretion as compared to basal glucose stimulation. Our results demonstrate that insulin secretory response of Chick B islets to Metformin and GABA is comparable to those of hIEqs. Moreover, both chick and hIEqs could be successfully cryopreserved and revived in a commercially available cryomix - Cryostore 5, indicating resemblance in their behaviour at sub-zero temperatures. **Conclusion** Present study advocates Chick islets as an alternative source for diabetes research and islet banking.

INTRODUCTION

Diabetes mellitus is a syndrome of disordered metabolism, and refers to the group of diseases leading to hyperglycemia due to defects either in insulin secretion (type1diabetes) [1], or in insulin action (type 2 and gestational diabetes [2, 3]. Diabetes and its treatments can cause many secondary complications such as hypoglycemia, ketoacidosis, cardiomyopathy, nephropathy, neuropathy etc. [4-9]. Previously we have shown that isolated islets from the dorsal lobe of 4-5 days old chick pancreas provide an excellent alternative to mammalian systems for diabetes research [10]. We also compared the response of chick and mouse islets to Streptozotocin (STZ) and showed that chick islets have better antioxidant defence, making them resistant to STZ [11]. Similarity between human and avian islets for insensitivity to STZ resulting in the failure of induction of experimental diabetes has been shown [12, 13]. In the light of these evidences the present study is undertaken to compare the insulin secretion by pancreatic islets of the members of these two phylogenetically closer groups in the animal kingdom. Our data endorse the suitability of chick B islets as an alternative model to mammalian islets for hypoglycemic testing and possible xenogenic transplantation.

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MATERIALS AND METHODS

Animals

One-day-old chicks of either sex were obtained from local market, in old Yehlanka, Bangalore, India. These were reared for 4 to 5 d in plastic baskets in a room. Temperature conditions were provided as per the requirement of warmth to the birds. Commercial feed available at the local hatchery was fed to the chicks. Freshwater supplemented with the antimicrobial antibiotic Enrocin (Emil Pharmaceuticals Industries Ltd., Tarapur, Thane, India) and multivitamin drops of Vimeral (Sundar Chemicals Pvt. Ltd., Chennai, India) at the recommended veterinary doses were made available to the chicks for 24 h.

Isolation of Chick B Islets

Five to six day old chicks were used for isolation of pancreatic islets by using protocol of Datar and Bhonde [14]. In short, dorsal ventral lobes of pancreata were aseptically removed and subjected to sequential digestion. Collagenase Type V (Sigma-Aldrich) 1mg/mL was prepared in Hanks' Balanced Salt Solution, containing Ca. The digestion mixture was centrifuged at 800rpm for 10 min. After 2 washings, the pellets were seeded in culture flasks (25 cm^2, Nunc A/S, Roskilde, Denmark) KO DMEM (Gibco Laboratories, Gaithersburg, MD), pH7.4, supplemented with 10% (vol/vol) fetal calf serum (Gibco Laboratories). Cultures were incubated at 37°C in a CO_2 incubator (Hera Cell 240 CO_2 Incubator, Thermo scientific, Carlsbad, California, USA). Islets were ready after 24 h of incubation.

Isolation and Expansion of Human Umbilical Cord Mesenchymal Stem Cells (hUCMSCs)

Human Umbilical Cord MSCs were isolated and propagated using earlier protocol [15]. Briefly, cord was washed with HBSS and incubated for 2 h at 37°C with cocktail of enzymes. Disintegrated tissue was then centrifuged by adding HBSS and serum at 2000 rpm for 10 min. The pellet was seeded in culture flasks (25 cm2, Nunc A/S, Roskilde, Denmark) containing KO DMEM supplemented with 10% human umbilical cordserum. It was observed that hUCMSCs have doubling time of 48h.

Differentiation of hUCMSCs to Human Islet Equivqlents(hIEqs)

Single cell suspension was prepared from the confluent cultures of hUCMSCs. Cells were seeded with defined serum-free medium containing 1.5% BSA and 1X ITS supplement (5 mg/L of insulin, 5 mg/L of transferrin and 5 mg/L of selenium). On day 4, 0.3 mM Taurine was added to the serum-free medium. On day 7 serum-free medium was supplemented with GLP and Nicotinamide. Every 48 h medium was removed, and cultures were observed under phase contrast microscope. Floating hIEqs were collected and assessed.

Characterization of Chick B islets and hIEqs

Islet mormhometric studies were carried on a sophisticated image analysis system (Nikon Eclipse TE 200-S, Chiyoda-Ku, Japan).

Assessment of Islet Viability using Trypan Blue Dye Exclusion Test

Freshly isolated chick B islets and hIEqs differentiated from hUCMSCson 10th day from induction of differentiation were collected and assessed for viability using trypan blue dye exclusion test. Briefly, 0.4% (wt/vol) Trypan blue was added to the suspension of islets for 5 minutes. Islets were then observed under the inverted phase contrast microscope (Olympus, Tokyo, Japan). Blue stained islets were scored as nonviable and the unstained were scored as viable islets. The viability was found to be 98% for both chick B islets and hIEqs.

Assessment of Islet Specificity using Diphenyl ThioCarbazone (DTZ) Staining:

Specificity of the Chick B islets and hIEqs of varying sizes was examined by islet specific stain DiphenylThioCarbazone (DTZ) [16]. DiphenylThioCarbazone staining was carried out by adding 10 μL of DTZ stock to islets suspended in 1 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) with N-2- hydroxy ethyl piperazine -N1-2- ethane sulfonic acid (10 mM; HEPES) and incubated at 37°C for 10 to 15 min. The stained islets looked bright red under the inverted microscope (Olympus, Tokyo, Japan). Non islet tissue remained unstained.

Immunocytochemical Characterization

Chick B islets and hIEqs differentiated from hUCMSCs were stained with islet specific markers viz. Isl-1, C-peptide and Glut-2 following the routine protocol. Briefly, Chick B islets and hIEqs were fixed for 20 min in 4% paraformaldehyde in chamber slides and treated with 0.1% Triton X-100 to permeabilize cell membrane. Islets were blocked at room temperature in 5% BSA solution for 30 minutes and incubated with primary antibodies Isl-1, C-peptide and Glut-2 over night at 4°C. Subsequently, islets were washed with PBS and incubated with FITC-conjugated secondary antibodies at room temperature for 2 hours. Slides were counterstained with 4', 6-diamidino-2-phenylindole (DAPI) for 2-3 min and fluorescent images were captured (Nikon) (Table 1).

Insulin Release Assay

Chick B islets and hIEqs differentiated from hUCMSCs were hand picked and triplicate groups of 4 to 6 islets each were placed in a single well of a 24-well plate (NuncA/S)each well containing 250μl Krebs-Ringer bicarbonate buffer (pH 7.4) supplemented with 10mM HEPES (now called KRBH) (pH = 7.4). The plates were then incubated at 37°C in a CO_2 incubator for 1 h. The supernatant was collected and stored at -20°C.These islets were then transferred to KRBH supplemented with 2.5mM and 16.7mM glucose for hIEqs differentiated from hUCMSCs and chick B islets respectively. After incubating them for 1 h at 37°C, the supernatant was collected and stored at -20°C. Secreted insulin was quantified using Human insulin ELISA kit (Mercodia, AB, Sweden).Statistical Analysis Results are expressed as mean±SEM.

Cryopreservation of Islets and Assessment of their Functionality

Islets (Chick B islets and hIEqs differentiated from hUCMSCs) were stored in Cryostore (CS-5), a commercially available reagent which has 5% of DMSO, for 30 days in Liquid Nitrogen at -196°C in programmed manner at the rate of 1°C/min. After 30 days Islets were revived and were checked for their viability and functionality by performing insulin release assay as described above. Secreted insulin was quantified employing Human insulin ELISA kit (Mercodia, AB, Sweden). Statistical Analysis Results were expressed as mean ± SEM.

DATA ANALYSIS

For the glucose stimulated insulin scretion (GSIS) studies, 4-6 islets were handpicked. The assay was performed

Table 1. Pancreatic Markers.

Pancreatic Markers	Antibody	Dilution	Company and Catalogue no	Purposes
Isl-1	Anti-Islet-1	1:500	Abcam-ab86501	Sandwich ELISA, Flow cyt, IHC-P,WB, ICC/IF
Gult-2	Anti-Glut-2	1:500	Abcam-ab85715	Flow cyt, IHC-FoFr, WB, ICC, IHC-P
C-peptide	Anti-C-peptide	1:500	Abcam-ab8297	Competitive ELISA, IHC-P, ICC/IF, RIA

in 24 well plates in triplicates. All the experiments were repeated three times (n=3) for the validation of the data. For the calculation of insulin secretion, average of all the three sets of experiment was taken. The data was then analyzed using graph pad prism, version 5. The stimulation index was calculated as the ratio of amount of insulin secreted by high glucose upon low glucose.

STATISTICAL ANALYSIS

All the data values were expressed as mean±S.E.M. Significance of differences among groups were determined by using one way analysis of variance followed by Dunnett's post-test or by Students unpaired T-test. P value summary (*)<0.05, (**)<0.01 and (***)<0.001 respectively compared to low glucose. Unless mentioned otherwise, n=3 across experiments. Graph pad prism- version 5 was used for statistical analysis.

ETHICAL CLEARANCE

The use of chick for islet isolation was approved by Institutional Animal Ethics Committee (IAEC).The use of human umbilical cord for research purposes was approved by Ethical committee of Manipal Hospital Bangalore, India after getting written consent from the parents, indicating no commercial use.

RESULTS

Characterization of Chick B Islets And hIEqs

Freshly isolated Chick islets after 24h in culture were found to be spherical in shape with well-defined boundary and varied sizes(80-150 μ). The ILCs obtained on 10thday after induction of differentiation from hUCMSCs, also appeared spherical in shape however they were larger in size (120-150μ) than that of Chick B islets(Figure 1 A and C).

Assessment of Islet Viability and DTZ Specificity

Chick B islets and hIEqs differentiated from hUCMSCs showed 95% and 98% viability after 48h of incubation. These were stained brick red with DTZ indicating positive staining for a zinc-chelating agent known to selectively stain pancreatic β cells because of their high zinc content. This confirms their identity as islets.The single cells formed during the differentiation process did not stain positive for DTZ (Figure 1 B and D).

Immunocytochemical Characterization of Chick B Islets and hIEqs:

Immunofluorescence staining of Chick B islets and hIEqs revealed immune-positivity of early pancreatic marker-ISL1 and β cell markers Glut 2 and C-peptide, confirming their identity as islets(Figure 2 A-I for Chick B islets and K-R for hIEqs).

Effect of Additives on Insulin Secretion of Chick B Islets and hIEqs

The islets were assessed for their insulin secretion status upon stimulation with basal (2.5mmol/L) and high glucose(16.7mmol/L). Since metformin improves glucose mediated insulin release and GABA enhances the survival of human islets, we assessed their effect on insulin secretion. Chick B islets secreted 0.08 μg/lt and 0.798 μg/lt (P<0.001) (Figure 3A) of insulin upon stimulation with basal and high glucose respectively. A comparable insulin secretion pattern was observed in hIEqs upon stimulation with basal and high glucose, 0.06 μg/lt and 0.78 μg/lt (P<0.01) (Figure 3B). In addition, both the islets when stimulated with metformin (100μg/ml) also showed similar results. Chick B islets released 0.707 μg/lt (P<0.01) (Figure 3A) of insulin while hIEqs secreted 0.84 μg/lt (P<0.001) (Figure 3B) of insulin. GABA on the other hand enhanced the insulin secretion by 0.821 μg/lt (P<0.001) (Figure 3A) and 2.1 μg/lt (P<0.001) (Figure 3B) in Chick B islets and hIEqs respectively.

Cryopreservation of Islets

Both Chick B islets and hUCMSC islets were stored at -196°C for 30 days. To test their functional status GSIS was carried out after revival on 30th day. After stimulation, the insulin secreted by Chick B islets at low glucose was 0.03μg/lt while at high glucose it was 0.201 μg/lt (P<0.001). On the other hand hUCMSC derived islets exhibited insulin secretion of 2.5 μg/lt with low glucose and38.4 μg/lt with high glucose (P<0.001) (Figure 4A and B).

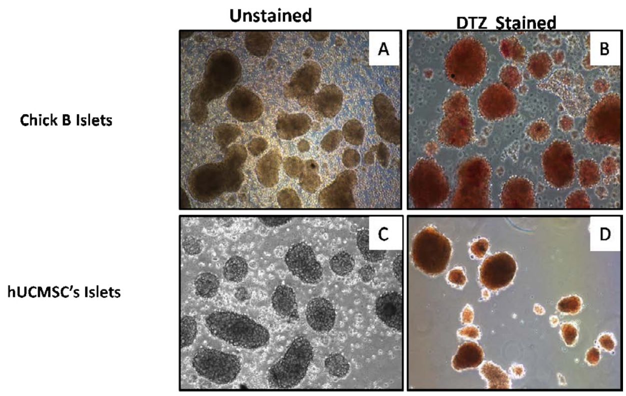

Figure 1. Figure 1A represents the unstained freshly isolated Chick B islets which are of two different sizes, medium and large (80-150μ and 100-150 μ). The brick red stain taken up by the Chick B islets as shown in figure 1B confirms their pancreatic status despite of their variation in sizes. Figure 1C demonstrates the unstained hIEqs generated from hUCMSCs while 1D represents DTZ stained hIEqs although the intensity of colour uptake is not similar for both the islets.

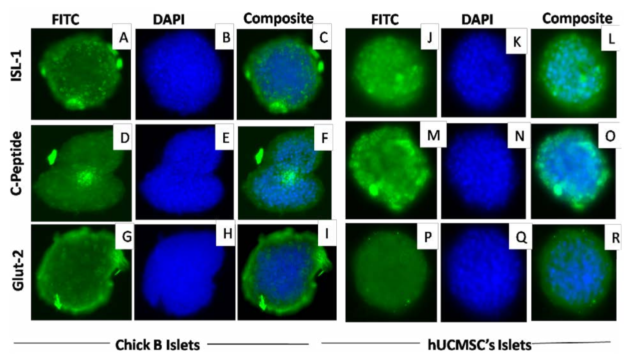

Figure 2. Chick B islets were positive for the pancreatic markers when immune stained with ISL1 (2A FITC, 2B DAPI and 2C, Composite), C-peptide (2D, 2E and 2F) and Glut 2(2G, 2H and 2I). On the other hand hUCMSC's islets were positively stained with the same markers, ISL1 (2J, 2K and 2L), C-peptide (2M, 2N and 2O) and Glut 2 (2P, 2Q and 2R).

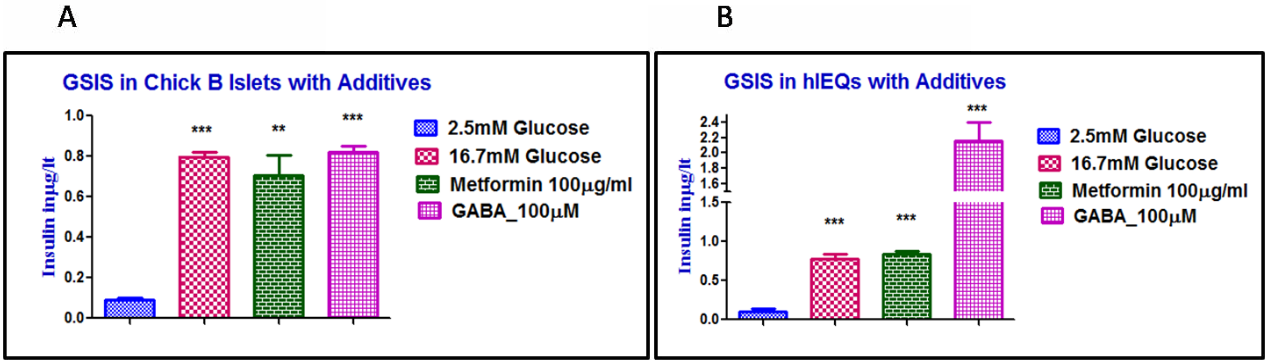

Figure 3. Figure 3A indicates insulin secretion pattern of freshly isolated Chick B islets upon stimulation with low and high glucose and the additives. Metformin and GABA showed similar insulin secretion as that of high glucose. The stimulation index was found to be 9.97, 8.83 and 10 upon stimulation with high glucose, metformin and GABA respectively. The response of hIEqs for insulin secretion was similar with high glucose and metformin, while GABA profoundly enhanced the insulin secretion (Figure 3B). The stimulation index was found to be 13, 14 and 35 upon stimulation with high glucose, metformin and GABA respectively

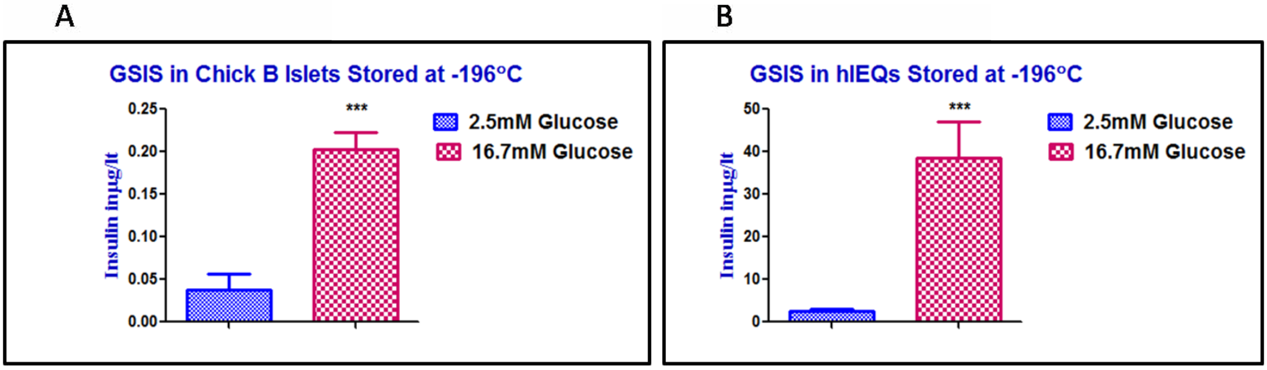

Figure 4. The Chick B islets maintained their functionality even after cryopreservation. The cryopreserved islets functionally behaved exactly like freshly isolated islets as insulin secretion status was similar upon revival (Figure 4A). The cryopreserved hIEqs showed immense increase in insulin secretion with high glucose (Figure 4B). The stimulation index for both Chick B islets and hIEqs were found to be 6.7 and 15 respectively.

DISCUSSION

Present investigation describes a comparative account of morphology, characterisation and insulin secretory response of chick B islets and hIEQ generated from hUCMSCs to various insulin secretagogues. Restrictions on the use of mammals for experimentation in diabetes research prompted us to look for alternative source of islets. In the present work we analysed the insulin secretory response amongst two homeotherms viz. chick and human. Our data reveals a striking similarity in insulin secretion response of chick and human islets. Similarities in their insulin secretory activity and sensitivity to STZ have been reported [12, 17, 18]. Here we studied islet specific markers and insulin secretion pattern of Chick B islets and hIEQ in response to various agents. Morphologically chick islets were found to be smaller in size than those of the hIEqs generated from hUCMSCs. However there was no difference in their viability. There are reports which suggest that islets smaller than 150 micron have higher stimulation index than the islets which are larger than 150 micron [19]. Although the islets derived from hUCMSCs were larger in size, they were found to be viable and their stimulation index was observed to be similar to that of chick B islets. Such size based difference in the functional analysis of the islets was not observed for the hIEqs. Immuno-cytochemical studies showed that the Chick B islets cross react with human antibodies for pancreatic markers- ISL1, Glut2 and C-peptide confirming the conservation of insulin secretion machinery between these two phylogenetically close groups of vertebrates. Both the islets were found to be positive for the pancreatic specific markers as revealed by immunofluorescence for ISL1, Glut-2 and C-peptide.

Freshly isolated chick B islets consist only of insulin producing cells and have a separate component of A islets of glucagon secreting cells which is a predominant feature of avian islets [20-22]. On the contrary the mammalian islets exhibit a mixture of both insulin and glucagon producing cells [23, 24], which has also been seen in hIEqs generated from hUCMSC's [15]. Insulin secretion in response to glucose stimulation by freshly isolated chick B islets was found to be similar to that of hIEqs generated from hUCMSC's. The most striking feature observed during the experiment was the consistent response of high glucose on insulin secretion by the chick B islets under various conditions. Animal to animal variation and the storage conditions did not affect the insulin secretary pattern of chick B islets with high glucose. However the same was not observed with hIEqs. The reason could be the different batches of hUCMSCs that were used for the generation of islets. The stimulation index of both the islets was found to be more than 8 in all the experiments.

Metformin is a widely used insulin sensitizer for the treatment of type 2diabetes mellitus. Metformin has been reported to protect rat islets from lipotoxicity and hence supplementation of metformin to high free fatty acid media improved the glucose-mediated insulin release [24]. The chronic exposure of high glucose to human islets leading to desensitization which was altered by metformin [26], gave us a clue to use this biguanide as insulin secretagogue. On the other hand GABA is an inhibitory neurotransmitter in mammalian central nervous system which is also known to inhibit glucagon secretion in the presence of glucose and thus indirectly increases insulin secretion. It has also been reported that GABA improves the survival of human islets and suppresses their immune cells [27]. Hence, we checked the effect of these agents on insulin secretion by both the islets. It was interesting to note that both Chick B islets and hUCMSC's generated hIEqs showed drastic increase in insulin secretion in the presence of Metformin and GABA.

We studied the behaviour of both these islets upon storage at -196°C (liquid nitrogen) in CS5 Cryostore which is a commercially available reagent. Revival of islets after 30days indicated 80% viability bytrypan blue dye exclusion test in both the types of islets (data not shown). Glucose stimulated insulin secretion by these islets indicated their functional status similar to those of freshly isolated/ generated islets indicating similarity in their response to a cryomix (CS5). Our studies indicate that the insulin secretion pattern of chick B islets is similar to the hIEqs. However, further studies are required for understanding the enzymatic makeup of Chick B islets and hUCMSC's generated IEQs to develop Chick B islets as promising alternative to mammalian islets for diabetes research.

CONCLUSION

The present study demonstrates for the first time a striking similarity in insulin secretion of Chick B islets and stem cell (hUCMSC) derived hIEqs in response to different secretagogues. It is noteworthy that metformin and GABA induce similar secretory response in both Chick B islets and hIEqs. This feature advocates Chick B islets as an alternative to hIEqs in large scale screening of hypoglycemics.

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Conflict of Interest

Authors declare no conflict of interest.

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