3D Culture is a Better Predictor of *in vivo* Drug Response than 2D Culture: A Growth Factor Study Comparative Review

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Review Article

ABSTRACT

Objective: To observe the effects of basic fibroblast growth factor and glial cell line-derived neurotrophic factor on 2D and 3D culture determining the cell survival rate, cell proliferation and insulin secretion function of pancreatic beta-cell (β-cell) spheroids following treatment with different doses of these growth factors *in vitro*.

Design: betaTC-6 cells cultured in monolayer and spheroid cultures were treated with three different doses of FGF-2 (10,100,1000ng/mL) and GDNF (100,200,400 ng/mL). A cell-based WST-8 assay was used to determine the proliferation and survival rate of the β-cell in both the culture models. Insulin ELISA was performed to determine the insulin secretion function *in vitro*.

Results: The results obtained on the 2D culture differ markedly from the results obtained on the 3D culture. Spheroids seeded at 500 and 1000 cells/spheroid showed improved cell proliferation when treated with 10 ng/mL FGF-2. However, this same dose of FGF-2 showed cytotoxic effects on 2D500 and 2D1000 culture. S500 spheroids treated with GDNF showed high cell proliferation at all doses of GDNF. 2D500 cultures showed slight improvement in cell proliferation at 100 and 400 ng/mL but did not show any such improvement at 200 ng/mL of GDNF.

Conclusion: Recently 3D culture models are gaining popularity in studies such as drug discovery and drug development for their ability to mimic the *in vivo* environment quite accurately. This study showed that 3D culture model is a better predictor of *in vivo* response to growth factors compared to the 2D culture model.

INTRODUCTION

Drug discovery and new drug development is an expensive and elaborate process. The main reasons for drug failure are inaccurate preclinical testing methods and inefficiency of the chosen in-vitro models. Insufficient data generation and poor predictability of the chosen models and methods lead to the termination of drug development process at a late pre-clinical stage. The use of new technologies in pre-clinical testing and in-vitro models may improve the success rate of drug development process through generating sufficient accurate data[1-3].

Cell-based assays are the invincible tools in drug discovery and development process. Although the traditional 2D cell cultures are predominantly used in HTS, 3D cell culture techniques are gaining rapid progress for applications in drug discovery.3D cultures have been used in different stages of drug discovery including disease modeling, target identification, validation, drug screening, potency profiling, target selection and toxicity assessment.The most effective cell-based assays with 3D cultures are cell viability, cell proliferation, signaling and migration.Different growth factors and their receptors play vital roles in the pathogenesis of pancreatitis. Studies on embryonic systems show evidence of the beneficial roles of basic fibroblast growth factor (bFGF or FGF2) in pancreatic development [4,5]bFGF is an FGF family member that is markedly overexpressed in human pancreatic carcinoma [6,7]. It binds with transmembrane receptors, which contain intracellular tyrosine kinase domains [8-12]. Glial cell line-derived neurotrophic factor (GDNF) produced by glial cells plays an important role in the development of the enteric nervous system [13,14].

Pancreatic cells share several biological characteristics with neuronal cells, including the expression of neuronal transcription factors [14-16] Several studies link GDNF to the survival of β-cells and the maintenance of their function. Increased expression of GDNF has been reported in the vicinity of pancreatic β-cells following islet injury, suggesting the involvement of GDNF in the survival and repair of islets [17-21]. Most studies on cell and tissue regulation have relied on the analysis of cells grown in two-dimensional (2D) cell-culture models, which fail to reconstitute the *in vivo* cellular microenvironment; these 2D cultures commonly do not maintain their differentiated functions [22]. To fully understand the formation and function of tissues, as well as their pathophysiology, it is crucial to study how cells and tissues behave as parts of whole living organs composed of multiple, tightly opposed tissue types that are highly dynamic and variable in terms of their three-dimensional (3D) structure, mechanical properties, and biochemical microenvironment. Although the development of one standard, simplified, *in vitro* 3D model suitable for biological and pathological investigations and drug discovery may not yet be feasible, standardized models for individual tissues or organs are a possibility [20-24]. The aim of the present study was to investigate the effects of bFGF and GDNF on the cell survival rate%, proliferation, and insulin secretion function of pancreatic β-cells *in vitro*, comparing the results obtained from the monolayer (2D) culture model with 3D culture model. This study also brings out the efficiency of the 3D culture model in accurately predicting the *in vivo* response to growth factors.

MATERIALS AND METHODS

Preparation of cell culture

The β-TC-6 cell line was purchased from the American Type Culture Collection (ATCC). The ATCC protocol was followed for initiating fresh culture on treated tissue culture dishes (100 × 20 mm; Corning, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, high glucose; Wako, Lot TWF 7025) supplemented with 15% foetal bovine serum (Gibco by Life Technologies, Lot 1927414), 1% glutaMAX-I (100X; Gibco), 1% sodium pyruvate (100X; Gibco), and 1% antibiotics (100X; Gibco, Lot 1924801), hereafter referred to as 'supplemented DMEM'. The cultures were maintained in a humidified incubator at 37°C in 5% CO_2. The medium was changed every 2 days. When the cells reached 80%–90% confluence, sub-culturing was performed.

Preparation of spheroids

When the cells reached 80%–90% confluence (Figure 1). The number of live cells was counted with a haemocytometer. According to the formula explained under 'appendix,' the volume of cell suspension needed for making spheroids (S500, S1000, S2000, and S4000) was prepared. To obtain the spheroids of different sizes, 500, 1000, 2000, and 4000 cells were seeded per pore of the 96 MP devices, for S500, S1000, S2000, and S4000, respectively. Then, the cells were seeded in 96 MP devices as shown in Figures 2-5 and placed in 96-well plates (Falcon,Tissue culture-treated, flat bottom, low-evaporation lid) for obtaining 3D culture samples according to the plate design depicted in Figure 6. After seeding, the 96-well plates were placed on a double shaker at 100–120 rpm for 10 min. The plates were then maintained in a humidified incubator at 37°C in 5% CO_2. Three days after seeding the cells, spheroids with the desired cell numbers were obtained.

Basic FGF treated samples

S500 0=spheroids made from 500 cells per spheroid seeding and no basic FGF was added.

S500 10=S500 treated with 10 ng/ml basic FGF.

S500 100=S500 treated with 100 ng/ml basic FGF.

S500 1000=S500 treated with 1000 ng/ml basic FGF.

2D500 0=500 cells seeded to form a monolayer culture and no basic FGF was added.

2D500 10=2D500 treated with 10 ng/ml basic FGF.

2D500 100=2D500 treated with 100 ng/ml basic FGF.

2D500 1000=2D500 treated with 1000 ng/ml basic FGF.
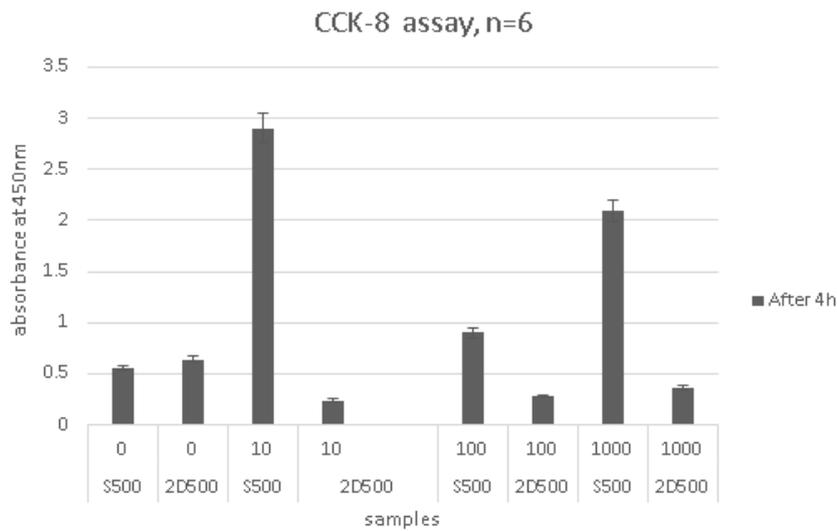
Figure 1. Basic FGF treated samples.
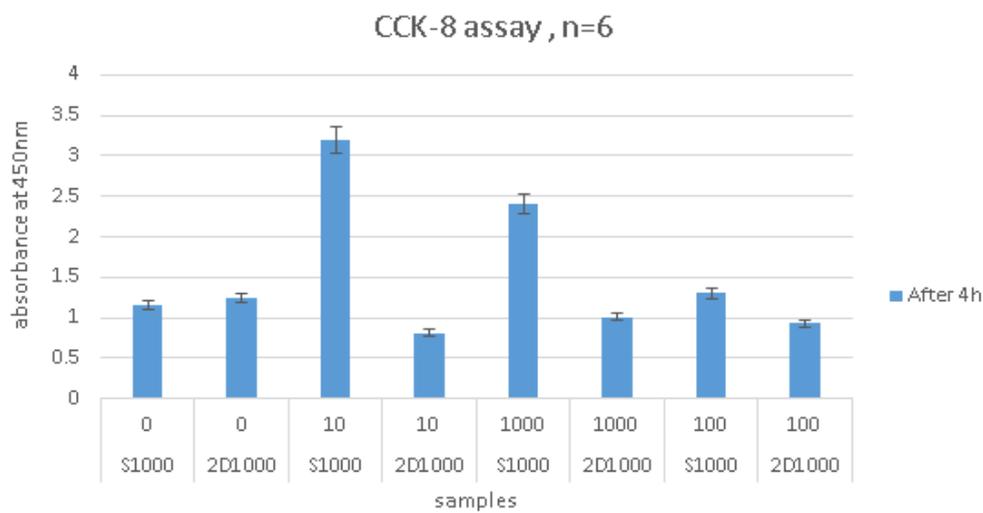
Figure 2. Basic FGF treated samples.
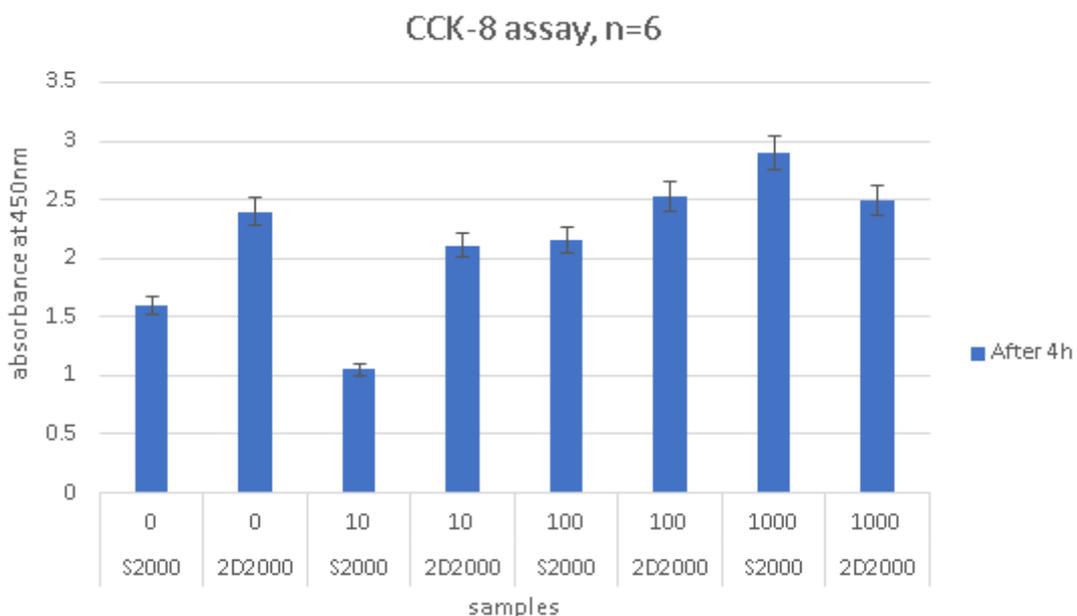
>3.5 Values are not included in the graph, shown as blank.

Figure 3. Basic FGF treated samples.
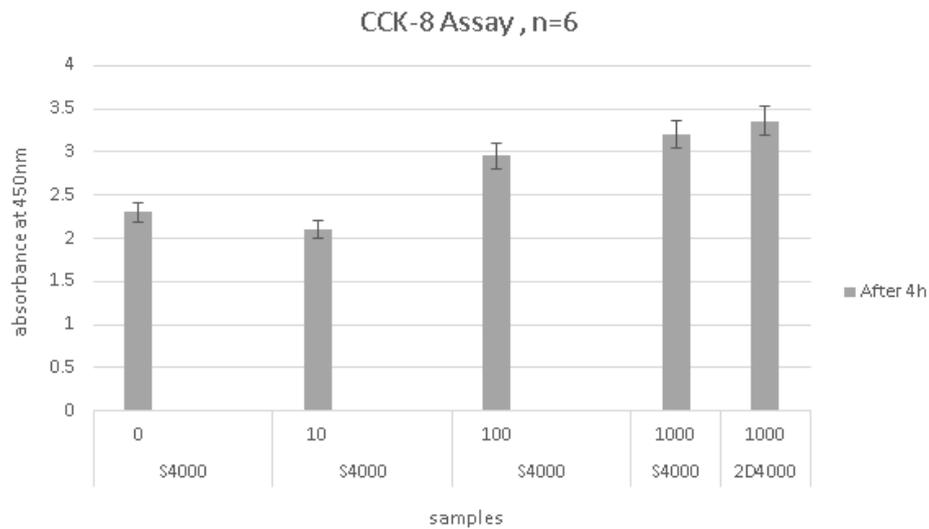
Figure 4. Basic FGF treated samples.

GDNF treated samples (Figures 5-8)

S=spheroid

2 D=monolayer culture

500, 1000,2000,4000 are no. of cells seeded.

0, 100, 200, 400 ng/ml are the doses of GDNF tested.
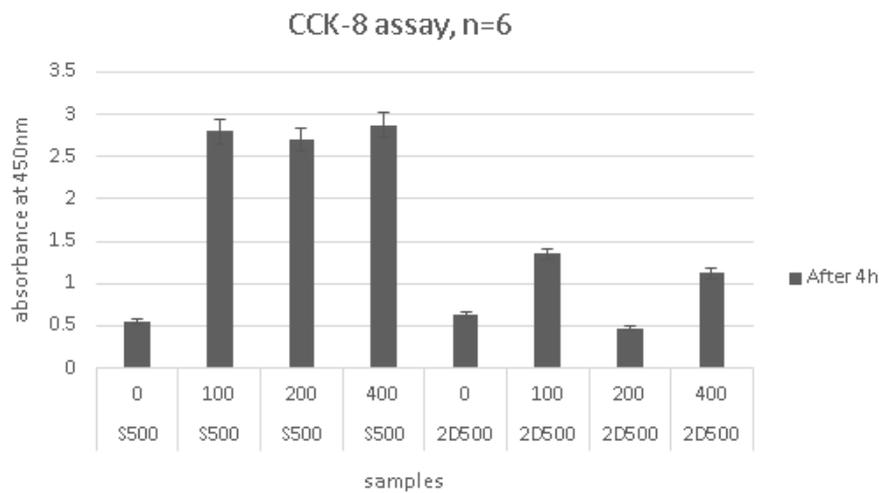
Figure 5.GDNF treated samples.
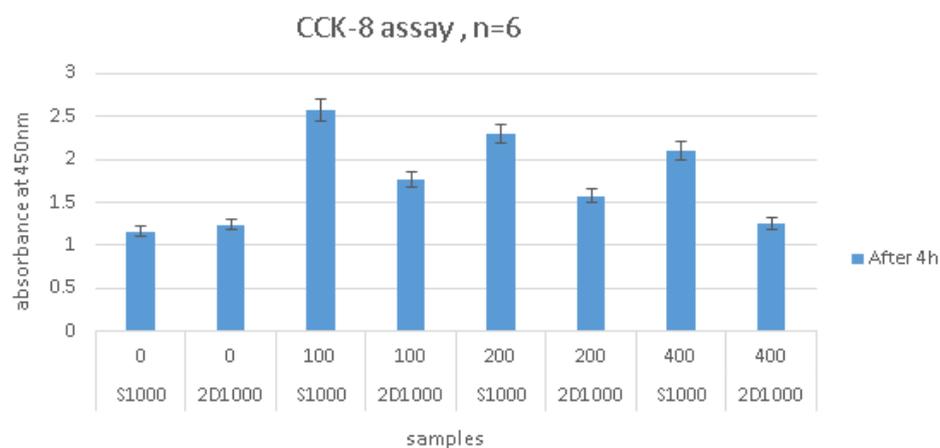
Figure 6. GDNF treated samples.

Preparation of 2D culture samples

For the preparation of 2D culture samples, the same procedure for the preparation of spheroids was followed, except for one step, i.e. the cells were seeded without MP devices directly into the 96-well plate according to the plate design selected. The groups were 2D500, 2D1000, 2D2000, and 2D4000.

Treatment with bFGF

bFGF (Recombinant human FGF-basic) (154 a.a.) (Catalogue # 100-18B, Lot# 041808) was purchased from Peprotech (USA). Reconstitution was performed according to the manufacturer's instructions. On day 3 of spheroid formation, the spheroids were harvested and centrifuged at 10 Xg for 1 min at 22–23°C. The supernatant was carefully discarded. Fresh complete medium with the respective doses of bFGF to be tested was used to resuspend the spheroids gently. Then, the spheroids were added to the respective 96-well plates and maintained in a humidified incubator at 37°C in 5% CO_2 for 24 h. For 2D cultures, the medium was replaced with fresh complete medium containing the respective doses of bFGF, according to the plate design, and the culture was maintained in a humidified incubator at 37°C in 5% CO_2 for 24 h.

Treatment with GDNF

Recombinant human GDNF (Catalogue #450-10, Lot #0606B64) was purchased from Peprotech. Reconstitution was performed according to the manufacturer's instructions. On day 3 of spheroid formation, the spheroids were harvested and centrifuged at 10 Xg and 22–23°C for 1 min. The supernatant was carefully discarded. Fresh complete medium with the respective doses of GDNF to be tested was used to resuspend the spheroids gently. Then, the spheroids were added to the respective 96-well plates and maintained in a humidified incubator at 37°C in 5% CO_2 for 24 h. For 2D cultures, the medium was replaced with fresh complete medium containing the respective doses of GDNF according to the plate design, and the culture was maintained in a humidified incubator at 37°C in 5% CO_2 for 24 h.

Cell viability assay

The detection sensitivity of CCK-8 (WST-8) is higher than that of other tetrazolium salts such as MTT, XTT, MTS, or WST-1. Therefore, we used CCK-8 for the cell viability assay. The medium was replaced with fresh complete medium containing CCK-8 reagent (Dojindo Molecular Technologies) according to the manufacturer's protocol. The culture plates were then incubated for an hour, and the absorbance was read at 450 nm wavelength with a Bio-Rad microplate reader using the microplate manager software version 6. Pathlength correction of 100 µL was applied, and step-wise reading was performed. The absorbance was read after 4 h. As WST-8 formazan is water soluble, it does not form crystals like MTT. Therefore, after 4 h of incubation with CCK-8 solution, measurement of optical density (OD) at 450 nm correlates with the number of viable cells [24]. The cell survival rate (%) was calculated according to the following formula:

Survival rate (%)=[Asample − Ab] / (Ac – Ab) × 100 Asample=Absorbance of the sample

Ab=absorbance of the blank

Ac=absorbance of the negative control.

***In vitro* insulin secretion from 2D culture**

2D cultures (2D500, 2D1000) were seeded onto a 24-well plate and maintained in a humidified incubator at 37°C in 5% CO_2. After the cells reached 80%–90% confluence, they were washed with glucose-free Krebs/HEPES Ringer solution [115 mMNaCl, 24 mM $NaHCO_3$, 5 mMKCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 25 mM HEPES (pH 7.4)] thrice and pre-incubated with glucose-free Krebs/HEPES Ringer solution at 37°C for 30 min. Then, the cells were incubated in Krebs/HEPES Ringer solution containing 1 mg/mL of bovine serum albumin and glucose (normoglycaemic, hypoglycaemic, and hyperglycaemic conditions) for 1 h. An aliquot (100 µL) of the supernatant was collected for ELISA. The amount of insulin released was measured with a mouse insulin ELISA kit (Mercodia Mouse Insulin ELISA kit, 10-1247-01, Lot no. 27936). The results are expressed in picomoles as the means ± SEM of at least two independent experiments (**Figures 7-10**).
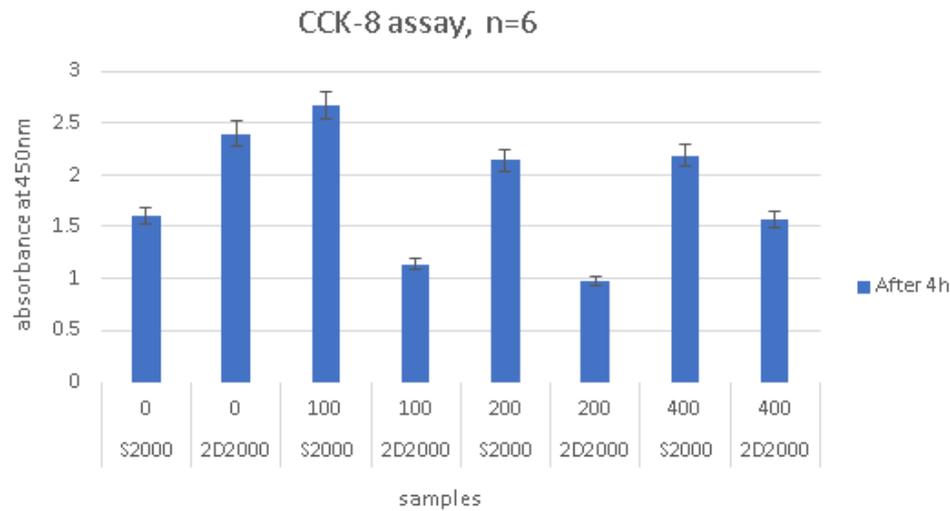
Figure 7. GDNF treated samples.
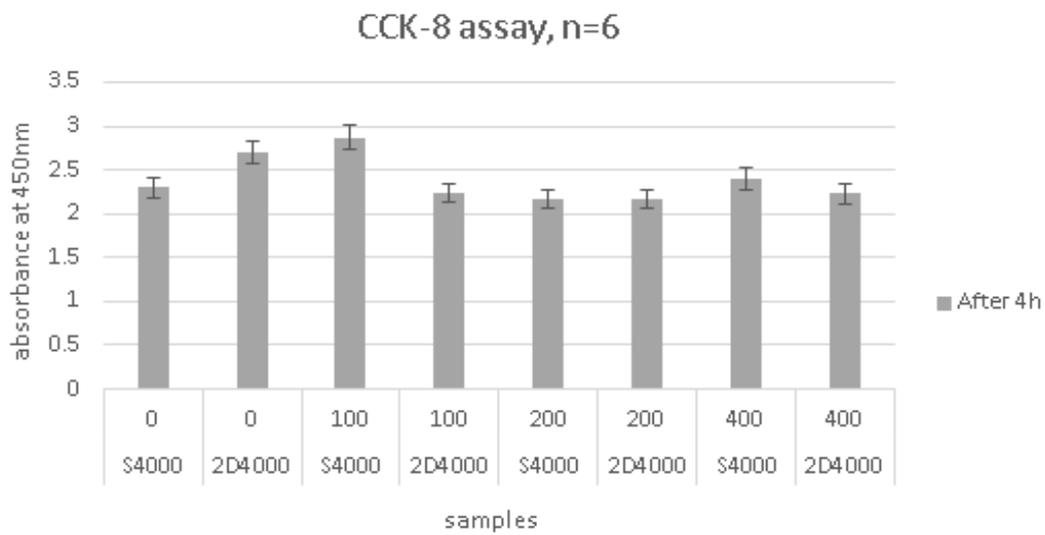
Figure 8. GDNF treated samples.
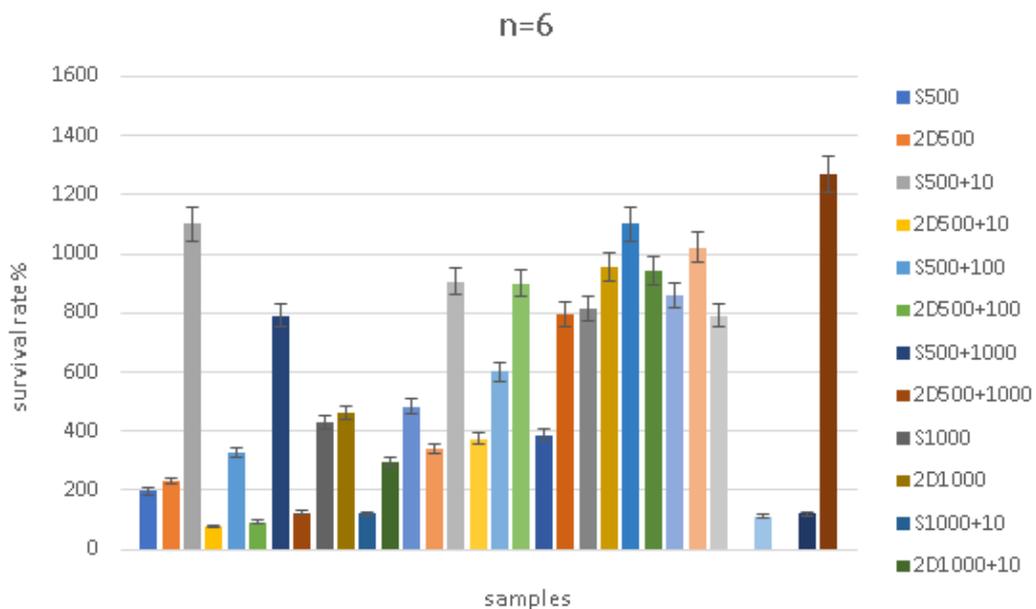
Figure 9. In basic FGF treated samples, this is how the survival rate looks like comparing 2 D and 3 D cultures.
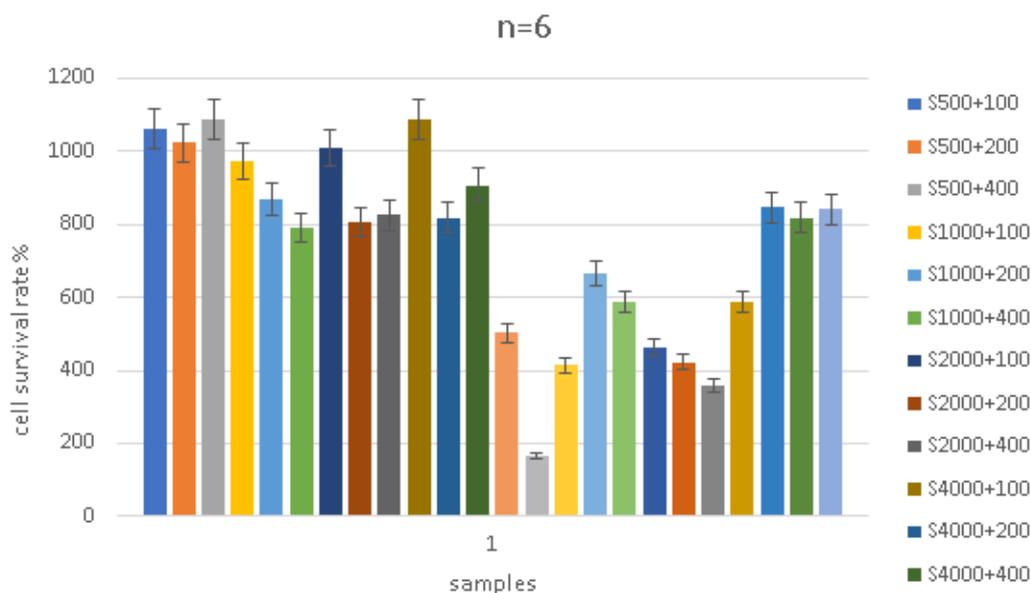
Figure 10. In GDNF treated samples, this is how the survival rate.

In vitro insulin secretion from spheroids

The spheroids were harvested, and 20 ± 2 spheroids were placed in 24-well plates and allowed to attach. Then, they were washed with glucose-free Krebs/HEPES Ringer solution thrice and pre-incubated with glucose-free Krebs/HEPES Ringer solution at 37 °C for 30 min. Next, the cells were incubated in Krebs/HEPES Ringer solution containing 1 mg/mL of bovine serum albumin and glucose (3.37, and 16.7 mM) for 1 h. An aliquot of the supernatant was collected for ELISA. The amount of insulin released was measured with Mercodia Mouse Insulin ELISA kit). The results are expressed in picomoles as the means ± SEM of at least two independent experiments.

Statistical analysis

All data expressed as mean ± SEM. One-way ANOVA was used to determine statistical significance. Differences were considered significant at $P < 0.05$.

RESULTS

Spheroids seeded at 500 and 1000 cells/spheroid showed improved cell proliferation when treated with 10 ng/mL bFGF. However, this same dose of bFGF showed cytotoxic effects on 2D500 and 2D1000 culture (i.e., the monolayer culture seeded at 500 cells/well and 1000 cells/well on a 96-well plate). Higher doses of bFGF (100 and 1000 ng/mL) showed cytotoxic effects on S500 spheroids but improved cell proliferation in S1000 spheroids. Cell survival rate (%) was high in S500 and S1000 groups treated with 10 ng/mL of bFGF. S1000 group treated with 1000 ng/mL bFGF showed higher cell survival rate than the 2D cultures. The insulin secretion function was maintained in the S500, S1000, 2D500, and 2D1000 groups treated with different doses of bFGF, with no or minimal significant difference from the non-treated groups. The highest cell proliferation in S2000 group was observed when treated with 1000 ng/mL of bFGF. S4000 showed high cell proliferation when treated with 1000 ng/mL of bFGF. 2D4000 treated with 1000 ng/mL of bFGF showed even higher cell proliferation. Insulin secretion function was maintained in all the groups, but no significant increase in insulin secretion was observed. This indicates that bFGF significantly contributes toward cell viability, cell proliferation, and cell survival in β-cell spheroids but does not play a significant role in the insulin secretion function of β-cell spheroids.

S500 spheroids treated with GDNF showed high cell proliferation at all doses of GDNF. 2D500 cultures showed slight improvement in cell proliferation at 100 and 400 ng/mL but did not show any significant improvement in cell proliferation at 200 ng/mL of GDNF. The S1000 group showed the highest proliferation at 100 ng/mL of GDNF. In the 2D1000 culture group, the highest proliferation was observed at 100 ng/mL. In the S2000 and S4000 groups, treatment with 100 ng/mL of GDNF showed the maximum proliferation. However, no significant proliferation was observed in the 2D2000 and 2D4000 groups treated with any dose of GDNF. All the spheroid groups showed the highest cell survival rate when treated with 100 ng/mL of GDNF, while the 2D culture groups failed to show significant improvement in cell survival rate. The S500 group treated with GDNF did not show improvement in insulin secretion, but the S1000 group maintained the insulin secretion function in hypoglycaemic, normoglycaemic, and hyperglycaemic conditions *in vitro*. No significant increase in insulin secretion function was observed in any group. 2D cultures showed higher insulin secretion than the spheroid culture groups.

DISCUSSION

The advantages of 2D culture models lie in their low-cost maintenance and performance of functional tests. But the disadvantages are also varied. It is now well-known that cell-cell interactions and cell-extracellular interactions are not accurately represented in a 2D models and that these interactions are crucial for cell differentiation, proliferation, viability, drug metabolism and responsiveness to stimuli.

When isolated from the source of origin and transferred to a 2Denvironment, cellular morphology undergo alteration an so does the mode of cell division. There is a loss of diverse phenotype. These changes affect the function, organization of structures inside the cell, secretion and cell signaling. Loss of polarity changes the response of those cells to phenomenon like apoptosis, etc.Also, cells in a monolayer are exposed unlimitedly to the ingredients of the medium. Although *in vivo*, the tissues are least likely to be exposed to the nutrients in this manner.The concept of 3D models was built to culture the cells from the donor's tissues in multicellular three-dimensional structures that would imitate the architecture of the parental tissue more accurately. This model ensures an improved cell-cell as well as cell-extracellular environment interaction. The morphology, polarity and cellular topology is maintained in such models.

Spheroids are scaffold-free based 3D cultures with a variety of properties like a gradient for efficient diffusion of growth factor, etc. the size of the spheroid depend on the number of cells seeded and can be increased to a size where they show oxygen and nutrient gradients similar to target tissues. Spheroids can easily be analyzed by imaging using light fluorescence and confocal microscopy. The problem of low reproducibility in 3D culture was solved by Vinci et al., where they created one spheroid per well in a 96 well ultra-low attachment plate [REF]

In this study, using the device developed by our lab, we are able to create 10 ± 2 spheroids per well in 96 well ultra-low attachment plates, which contributes to cost-reduction and improved reproducibility. 3D models, due to their ability to mimic native tissue environment, are now gaining popularity in drug discovery studies. A multitude of studies have consistently indicated that gene expression profiles, cellular phenotypes, differentiation capabilities, and functionalities are affected by tissue architecture. Thus, the drug evaluation process will benefit immensely from the accurate predictions of cellular responses displayed by 3D-engineered tissue models when exposed to drugs of interest *in vitro* [21]. Cells in monolayer are equally exposed to the growth factors in the medium, but in the spheroids, the growth factors may not be able to reach the cells near the core. Moreover, cells in monolayer tend to be in the same stage of cell cycle whereas in the spheroids, some cells may be in the proliferating stage, some may be quiescent, some necrotic or hypoxic. In the 3D spheroids, cells seem to show more resistance to the drug, but these models better predict the *in vivo* drug response.

The present study was conducted to determine the effects of bFGF and GDNF on the survival rate, proliferation, and insulin secretion function of pancreatic β-cell spheroids treated with different doses of these growth factors *in vitro*. These results were also compared between 2D and 3D cultures.

By inducing the synthesis of proteinases, FGF-2 promotes angiogenesis [9], endothelial cell migration and DNA synthesis [10], and *in vitro* capillary tube differentiation [11]. In this study it was found that FGF-2 increased the proliferation, viability, and insulin secretion function of pancreatic β-cells. But 2D cultures show cytotoxic effects to doses which otherwise show positive results on the 3D cultures.The overexpression of GDNF in glial cells increases β-cell survival and improves glucose tolerance in transgenic mice [12,15-18]. GDNF has shown improved post-transplantation graft function in human islet grafts. It has shown potential to treat Parkinson's disease before. GDNF produced in the pancreas acts as a neurotrophic factor for gut-resident neural progenitor cells. It may be a therapeutic target for increasing β-cell mass. Despite GDNF's beneficial role in pancreatic development, its potential in the regeneration of β-cells has not yet been explored.

CONCLUSION

While this study demonstrates the usefulness of FGF-2 and GDNF in type 1 diabetes mellitus treatment, further detailed *in vivo* studies are necessary to validate these findings. 2D cultures do not accurately replicate the *in vivo* situation. Consequently, we observed an inconsistency between the results obtained from 2D cultures and 3D cultures. 2D and 3D cell culture models are copiously used in the field of pharmaceutical research and development. Despite its limitations, 2D cultures are still in practice worldwide but are recently being considered as inaccurate and unreliable models.

3D cell cultures promise to bridge the gap between traditional 2D culture and *in vivo* animal models. However, an ideal 3D model still does not exist. In this study the WST-8 assay showed different findings for the proliferation of cells in the two different types of cultures and the results obtained on the 3D cultures were closer to the expected *in vivo* cellular response. This study proves that 3D models have more potential in the study of new biomarkers and new treatment strategies leading us to an ultimate aim of personalized medicine. Further studies can improve the reproducibility, high throughput analysis and compatibility to demonstrate uniformly standardized and validated 3D models, that will help identify relevant efficacy and toxicity data in drug discovery.

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