

## The Effects of Cord Blood Serum on Survival of Rat Pancreatic Islets during *In vitro* Culturing

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### Abstract

**Background:** Transplantation of islets of Langerhans can improve glycometabolic control in type 1 diabetic patients; however, recipients usually require islets from 1-3 donor pancreas. Current isolation techniques generally recover <50% of the islets from a given pancreas. This study aimed to determine the effect of cord blood serum (CBS) on isolated pancreatic islet cells *in vitro*.

**Methods:** Rat pancreatic islets were isolated via collagenase digestion, and then cultured with and without the presence of 50  $\mu$ l, 100  $\mu$ l, and 150  $\mu$ l of CBS. Next, islet cell viability was determined using a fluorescein diacetate and propidium iodide mixture via fluorescence microscopy. Additionally, islet function was evaluated based on insulin secretion.

**Results:** Treatment of primary cultures of rat pancreatic islets with CBS resulted in augmented  $\beta$ -cell viability. It was observed that the viability rate increased as the concentration of CBS increased and that viability was higher in the 150  $\mu$ l of CBS group than in the control group. CBS was observed to preserve  $\beta$ -cell glucose responsiveness and insulin secretion under basal (3.3 mmol<sup>-1</sup>) and stimulated (16.7 mmol<sup>-1</sup>) glucose conditions. Moreover, a significant increase in insulin secretion was observed following treatment with 50  $\mu$ l of CBS.

**Conclusions:** The present findings show that cord blood serum treatment of freshly isolated rat islets had a positive effect on islet survival by increasing their viability. We think these findings suggest that CBS might be a suitable factor for optimizing and stimulating recovery, and subsequent function of islets during *in vitro* culturing, indicating that CBS might play an important role in the biophysiological function of pancreatic islets.

**Keywords:** Islet cell; Cord blood serum; Diabetes mellitus

### Introduction

$\beta$ -cell replacement via transplantation of whole organ or islet cells is currently regarded as an acceptable therapeutic option for patients with type 1 diabetes; both methods have a positive effect on the achievement and maintenance of good glycemic control [1]. Islet cell transplantation has clinical indications similar to those of the whole organ procedure and is a more modern and highly specialized technique that is less invasive, but it requires generally multiple donor organs for transplantation of an adequate number of islets. This multiple donor requirement is a major limiting factor to its widespread use because of the shortage of donor organs [2]. In addition, an immunosuppressive regimen is required to avoid graft rejection. Apart from immune rejection, another critical problem is islet cell survival in a new environment. In fact, shortly after implantation islet grafts function poorly and many transplanted  $\beta$ -cells undergo apoptosis prior to full engraftment [3].

It is estimated that only 30% of an islet's mass is stably engrafted, despite administration of a large quantity of islet cells; therefore,

instead of increasing the number of islet cells implanted, a better strategy would be to improve islet graft survival and proliferation potential during the pre-transplantation culture period and the days immediately following transplantation [4]. Another important factor to consider is the isolation procedure and the possibility that a high percentage of islet cells might be damaged during this phase. Despite recent improvements to the isolation technique, such as the two-layer method and a less toxic iodixanol gradient, it is possible to isolate a sufficient number of islet cells for transplantation in only 50% of the procedures performed [2]. Furthermore the apoptotic and necrotic processes, that occur in the early post-isolation period, damage a significant number of islet cells [5,6]. As such, identification of molecules that can promote both  $\beta$ -cell survival and function would be of great relevance to the design of new therapeutic strategies aimed at improving  $\beta$ -cell function and increasing the number of islet cells for transplantation. [7,8] CBS can be considered the human equivalent of fetal bovine serum (FBS), because both are derived from blood obtained during the highly proliferative phase of early development. Growth factors in CBS are likely to be present in higher concentrations than in adult serum, so as to facilitate fetal growth and development. It was recently reported that CBS had a significantly higher

concentration of growth factors [epidermal growth factor (EGF), hepatocyte growth factor (HGF), fibroblast growth factor 6 (FGF-6), platelet-derived growth factor (PDGF), insulin-like growth factor binding protein (IGFBP-1, IGFBP-2 and IGFBP-6)] and cytokines [brain-derived neurotrophic factor (BDNF), growth-related oncogene (GRO), and Leptin] than adult serum and FBS [9,10]. Growth factors EGF, HGF, FGF-6, PDGF, IGFBP-1, IGFBP-2, and IGFBP-6 have been shown to promote the proliferation and differentiation of different types of cells [11-15]. The high concentrations of these growth factors and chemokines in CBS may be explained why CBS could be used to support cell proliferation. In the light of these reports, the present study aimed to determine the effect of CBS on isolated pancreatic islet cells *in vitro*.

## Materials and Methods

### Experimental animals

The study included 30 adult male Wistar albino rats weighing 200-250 g. Male wistar rats, which were housed in a room maintained at 12-hour light-dark cycles with a constant temperature of  $22 \pm 2$  C and animals feed ad libitum. The study was conducted at Diskapi YB Teaching and Research Hospital, Animal Laboratory, Ankara, Turkey, and the study protocol was approved by the animal ethics committee (protocol 2012-2035).

### Islet cell isolation

Immediately after pancreas were removed from the rats, enzymatic incubation was performed in a 37°C water bath for 18 min. After repeated centrifugation, 500 cells were transferred into 15 mL falcon tubes via handpicking using an inverted microscope, centrifuged at 1200 rpm, and then washed twice with RPMI 1640 medium (Lonza, BE12-167 F, Netherlands). Islet cells were stained with fluorescein diacetate (FDA)/propidium iodide (PI) for viability assessment. The relative percentage of PI-positive cells to FDA-positive cells facilitated semiquantitative analysis of islet cell viability. The mean viability value was analyzed using the MATLAB program (MathWorks, USA). Islet cell purity was assessed under a light microscope via dithizone (DTZ) staining (Sigma-Aldrich, D5130, USA).

### Islet cell culturing

Islet cells were divided into 4 groups; a control group that included only islet cells, and 3 other groups treated with 50 µL, 100 µL, and 150 µL of CBS RPMI (+) medium. After 48 h in culture medium islet cell samples from each of the 4 groups were collected, and viability testing with FDA/PI fluorescent staining was performed. Islet cell function was evaluated using the glucose stimulation test. Islet cells cultured with and without CBS were stimulated with 2 different glucose concentrations ( $3.3 \text{ mmol}^{-1}$  and  $16.7 \text{ mmol}^{-1}$ ) in isolation medium, and then incubated for 45 min at 37°C. Insulin release was measured on supernatants by using Ultrasensitive Rat Insulin ELISA (Millipore, USA), according to the manufacturer's recommendations. Results are presented as  $\text{pg ml}^{-1}$  after normalization with total protein content.

### Cord blood serum preparation

Cord blood was obtained from patients undergoing routine obstetric delivery via cesarean section. Exclusion criteria for cord blood collection were an infectious disease in the mother or fetus, and a history of using maternal cord blood for therapeutic purposes. Cord

blood was collected at the time of delivery under aseptic conditions. After clamping and cutting the umbilical cord, the blood from the placental side of the umbilical cord was collected into a sterile collection bag. The blood was then allowed to clot at 4°C for  $\geq 4$  h. Serum was then decanted away from the clot and processed via centrifugation at 1200 g for 20 min to separate the serum from remaining cells. The supernatant was collected and sterilized with a 0.2 µm filter. Serum was then stored in sterile tubes at 80°C and was used for the experiments within 6 months of storage.

### Statistical analysis

Data were analyzed using SPSS v.15.0 (SPSS, Inc., Chicago, IL). Findings are shown as frequency and percentage. As a result of the normality test, the Kruskal-Wallis H test was used to compare variables not normally distributed between  $>2$  groups. The Wilcoxon signed-rank test was used to determine the difference in cell viability between measurement times. The level of statistical significance was set at  $p > 0.05$ .

## Results

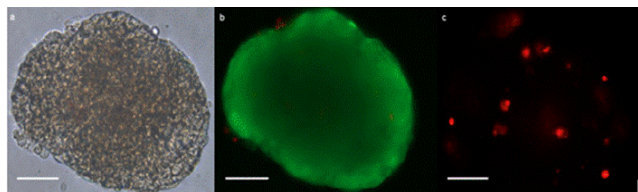
### Evaluation of viability via the fluorescent staining test

Islet cell viability was determined via FDA/PI staining of fresh islet cells and islet cells treated with CBS in culture medium for 48 h. Mean viability of fresh islet cells was  $87.959\% \pm 2.725\%$  and mean viability of cultured islet cells at 48 h was  $85.981\% \pm 3.390\%$ . Cultured islet cells had lower viability than fresh islet cells, but the difference was not statistically significant.

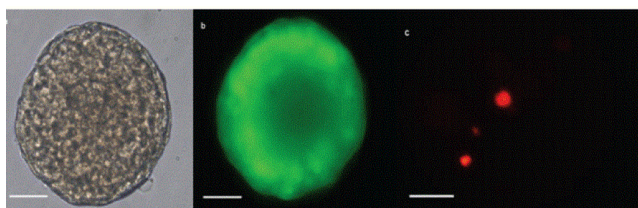
In the study, the viability levels of the control islets and the islets cultured by applying 3 different doses of cord serum were examined with PI/FDA staining using MATLAB program (Figures 1-4). The experiment was finalized after repeating for a total of three times. The fresh viabilities of the obtained islets were evaluated. Afterwards, the islets were incubated in culture environment by adding 50 µL, 100 µL, and 150 µL cord serum and without adding cord serum for 48 hours and viability measurements were repeated and compared. Viability was significantly higher in the islet cells cultured with 150 µL of CBS, as compared to the control group ( $p < 0.05$ ). Although there wasn't a significant difference in the viability percentage between the control group and the treatment groups, the viability percentage increased as the CBS concentration increased (Table 1 and Figure 5).

	Mean	Median	Min	Max	SS
Control	85,98	85,48	79,18	90,93	4,29
CBS 50 µl	87,71	88,93	82,57	90,58	2,72
CBS 100 µl	88,72	88,74	84,70	92,08	2,20
CBS 150 µl	90,03	90,46	83,38	93,33	3,20

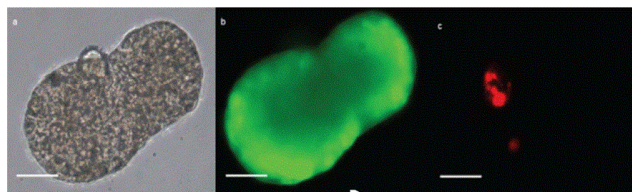
**Table 1:** Islet cell viability percentages based on FDA/PI staining and MATLAB analysis.



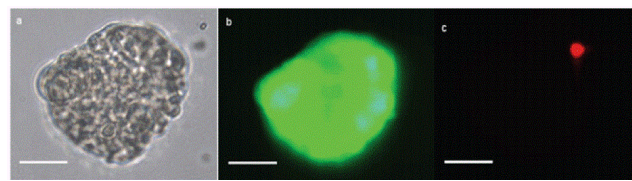
**Figure 1:** Islet cell viability in the control group (89.496%); a) Light transmitted microscopy image; b) Fluorescein-diacetate staining; c) Propidium iodide staining. Scale bars, 100  $\mu$ m.



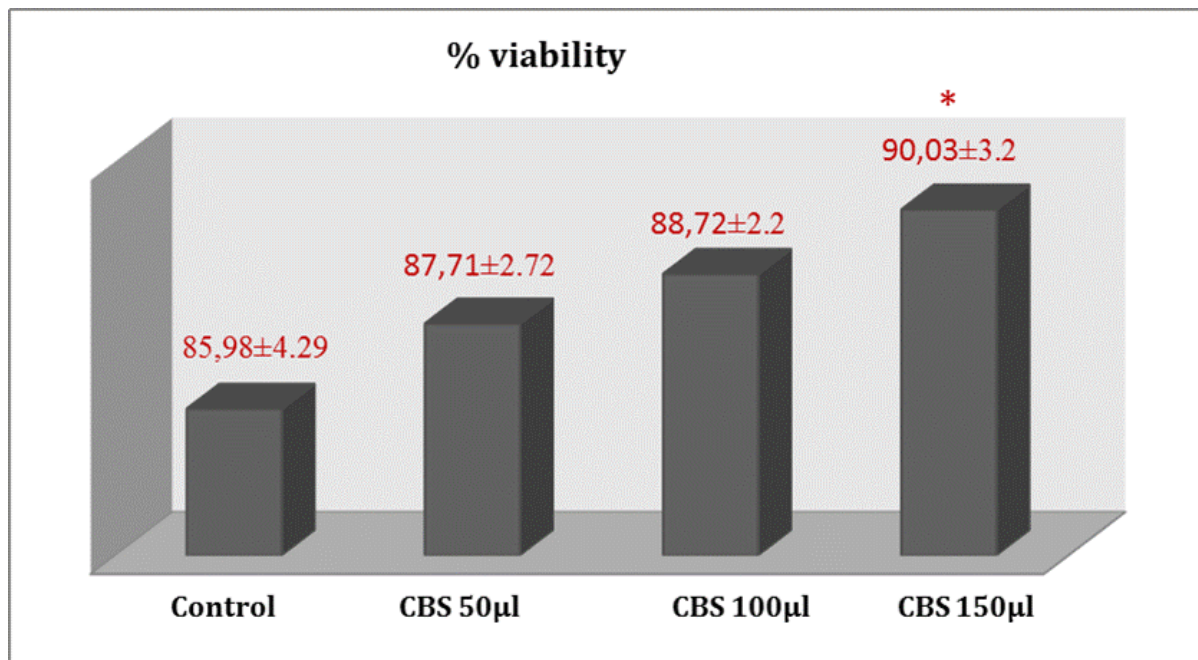
**Figure 2:** Islet cell viability in the 50  $\mu$ l of CBS group (95.693%); a) Light transmitted microscopy image; b) Fluorescein-diacetate staining; c) Propidium iodide staining.



**Figure 3 (a-c):** Islet cell viability after treatment with 100  $\mu$ l concentrations of CBS (95.94%); a) Light transmitted microscopy image; b) Fluorescein-diacetate staining; c) Propidium iodide staining.

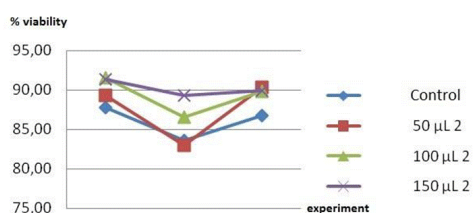


**Figure 4 (a-c):** Islet cell viability after treatment with 150  $\mu$ l concentrations of CBS (97.028%); a) Light transmitted microscopy image; b) Fluorescein-diacetate staining; c) Propidium iodide staining.



**Figure 5:** Islet cell viability percentages based on FDA/PI staining and MATLAB analysis.

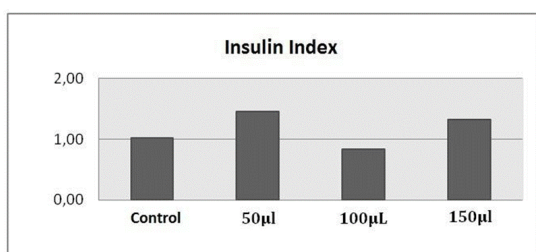
The study was conducted with 3 treatment conditions using 30 rats, with 10 rat pancreata included in each experiment. Each treatment condition yielded similar viability percentage findings, as shown in Figure 6.



**Figure 6:** Islet cell viability percentages for each treatment condition (N: 3).

### The glucose stimulation level

Insulin release in the control and 3 CBS groups incubated with high (16.7 mm) and low (3.3 mm) glucose concentrations were measured via ELISA. There weren't any significant intra-group differences between the low and high glucose concentration media findings ( $p > 0.05$ ), whereas the insulin stimulation index was significantly higher in the 50 µl of CBS group ( $p < 0.05$ ) (Figure 7).



**Figure 7:** The insulin stimulation index, according to group.

### Discussion

Diabetes mellitus (DM) is an epidemic disease that occurs in all age groups and is increasing in frequency worldwide. DM is associated with a decrease in quality of life and life expectancy, as well as with life-threatening complications, including chronic hyperglycemia, nephropathy, retinopathy, and neuropathy [16]. However, complications of DM could not be completely prevented despite tight glycemic control with intensive insulin therapy because natural regulatory skills of insulin-producing pancreatic beta cells could not be imitated with many available insulin formulations. Providing tight glycemic control gradually becomes more difficult because of progressive beta cell injury. This condition, today, has led to necessity of alternative or additional treatments [17]. Although pancreas transplantation enhance quality of life, reduce hyperglycemia-associated complications and prolongs survival, alternative treatment options are needed, as these benefits are accompanied by chronic

immunosuppression, surgical complications, and risk of graft rejection. Among such alternative approaches, pancreatic islet transplantation provides a significant advantage, because it is less invasive and protects patients against the complications of diabetes. Nevertheless, significant problems such as donor insufficiency, ineffective engraftment of transplanted tissue, and islet function loss in the long-term are encountered in clinical practice during islet transplantation [18]. This condition has led to trial of several methods that focus on preventing islet loss. In the experimental studies performed to protect islets from immune system, regions that are known to be protected from immune system have been chosen as the islet transplantation regions. Studies determined that these regions, in fact, provide protection for graft not keeping away the graft from immune system but rather synthesizing high amount of Fas ligand [19].

Another approach that has been tried for prolonging islet survival is encapsulation of islets prior to transplantation. Micro and macro-encapsulation method developed for this purpose may provide safe environment for islets with potential immune isolation and rich environment in terms of oxygen and food. Although islet encapsulation is promising, its usage has been restricted due to difficulties such as biocompatibility, ineffective immune protection, hypoxia, and post transplantation inflammation [20]. Insufficient donor tissue is another factor that influences islet survival. More than 60% of islet mass disappears within a few days after transplantation. Using a large islet mass instead of transplanting more than one islet leads remaining beta cell mass to be larger and thereby, more probably, to insulin independency [20].

Although many studies on how to increase the success of islet engraftment have been performed, significant loss of islet function and number during the peritransplantation period cannot be prevented, as yet [21]. The major obstacles for successful clinical islet transplantation are the isolation of sufficient mass of islets together with the management of graft rejection. The fatal outcome, which is not related to immune rejection, has been thought to be due to insufficient or non-established vascularization of transplanted islets [22-25]. It has been demonstrated that during the first two days after transplantation, islets are avascular, leading to processes that impairs the central b-cell mass of the islets [26,27]. As demonstrated [28,29] the main causative mechanisms involved in core cell damage might be necrosis or apoptosis. Necrotic cell death may depend on the limitation of nutrition diffusion, while apoptosis is generally caused by pathological atmosphere arising from the isolation procedure. The advantage of the pre-transplantation culture consists in practicing interventional strategies to prevent the profound b-cell loss occurring via apoptosis, which has been estimated to cause up to 70% of the transplanted b-cell mass destruction [30-32]. In consideration of these indications, use of protective factors that could increase beta cell viability and prevent apoptosis is under intensive investigation [4]. In this context, the present study aimed to determine the potential therapeutic effect of CBS during the culturing stage of the pre-transplantation period on islet viability and insulin release.

During pregnancy, cord blood transports nutrition from the mother to the fetus. The embryo transformation from a fertilized egg to a fetus is dependent on the nutrients in cord blood. As reported, CBS contains growth factors at much higher levels than adult blood serum. The levels of erythropoietin (EPO), granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), and colony stimulating factor-1(CSF-1) are higher in CBS than in adult blood serum [33-35]. Proteomic studies have shown that CBS

increased complement and coagulation cascade protein, cellular conduction protein, and extracellular matrix proteins levels, and cell proliferation and mitotic activity proteins [9]. CBS contains higher levels of growth factors (EGF, HGF, FGF-6, PDGF, IGFBP-1, IGFBP-2, and IGFBP-6) and cytokines (BDNF, GRO, and Leptin) than fetal bovine serum [10]. It was reported that BDNF prevents exhaustion of the pancreas in diabetic mice by maintaining the histologic cellular organization of beta cells and non-beta cells in pancreatic islets and restoring the level of insulin-secreting granules in beta cells [36]. In addition, it was shown that leptin increased viable cell numbers via suppression of apoptosis in isolated pancreatic islet cells under experimental conditions. This mechanism might account at least in part for an obesity-induced increase in pancreatic beta-cell mass [37].

HGF is a factor involved in regeneration after hepatic damage, which originates primarily from the mesangium. HGF has been observed in pancreatic islets and shown to be a mitogenic and insulinotropic agent *in vitro* fetal and human islet cell studies [38]. FGF was predicted to have beneficial effects on glucose homeostasis in diabetic animals, and the preservation of beta cell function and viability [39]. This property of CBS, which contains all these factors at higher levels than fetal bovine serum and adult blood serum, supports the hypothesis that CBS could increase the viability and insulin-releasing function of islets.

In the present study fluorescent microscopic measurement was performed to determine the viability of pancreatic Langerhans islet cells incubated in culture with CBS for 48 h and the FDA/PI stain uptake percentage was calculated using the MATLAB program. The glucose stimulation test was used to functional examination of islet cells obtained. In addition, islets insulin release with low and high glucose concentrations were measured.

Recently, many centers have introduced the culturing of human islets prior to transplantation [40-42] because it provides many benefits to clinical islet transplantation. *In vitro* culture may reduce islet immunogenicity by depletion of viable hematogenous and lymphoid cells and reduce exocrine contamination of transplanted tissue. Other benefits are additional quality control testing of isolated islets, initiating time-dependent immunosuppressive protocols, and it preserves the islets during travel time for recipients living far away from transplant centers. However, it is well documented that isolated islets deteriorate rapidly in culture for clinical islet transplantation [42-44] which negatively affects the transplantation success rate. Because of the benefits of the culture method, strategies and treatment methods for preventing islet loss in culture have recently been investigated.

In the present study the relationship between different CBS concentrations and islet cell viability was examined; conclusively, it was observed that the viability rate increased as the concentration of CBS increased and that viability was higher in the 150  $\mu$ l of CBS group than in the control group. Islet function was higher based on the insulin stimulation index, which was calculated according to insulin release in low and high glucose media. Although there was not a statistically significant difference in insulin release between the control group and the CBS groups, the insulin stimulation index was significantly higher in the 50  $\mu$ l of CBS group, which we think might have been due to the fact that CBS contains many growth factors and cytokines in non-standardized and different concentrations.

To the best of our knowledge the present study is the first to examine the effect of CBS added to culture on the viability and

function of pancreatic islets, and as such represents an important contribution to the literature. The present findings show that CBS added to culture after isolation preserved the viability of pancreatic islets. *In vivo* post-transplantation efficiency should also be evaluated and additional proteomics studies are needed to better understand the benefits of CBS-augmented culturing of islet cells.

### Author's Contributions:

BK, FAP carried out experiments; BK, FAP and TD designed the study and wrote the manuscript. GE analyzed data. EC contributed to the discussion. All authors have read and approved the final manuscript.

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