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Research Article

EFFECTS OF MEDIUM SUPPLEMENTS ON VIABILITY AND FUNCTIONALITY IN LONG-TERM CULTURED ISLET CELLS

Gülbahar Böyük [1], Ayşe Arzu Yiğit[2#*]

[1] *Ankara Medipol University, Faculty of Medicine, Department of Physiology, Ankara, Turkey*
[2] *Kirikkale University, Faculty of Veterinary Medicine, Department of Physiology, Kirikkale, Turkey*
[#]*Since completion of this research, Dr. Yigit's affiliation has changed to "Department of Physiology, Faculty of Medicine, Baskent University, 06790, Ankara, Turkey"*

**Corresponding Author:*
E-mail: aarzuyigit@baskent.edu.tr

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ABSTRACT. It is highly important to keep islet cells alive and functional before transplantation. This study aims to maintain the viability and functionality of islets with different supplements and provide longer incubation. Isolated islets were cultured with RPMI1640 with 10% fetal bovine serum (FBS) or 1% insulin-transferrin-sodium selenite (ITS) premix or 0.3% bovine serum albumin (BSA) up to 120h. Islets were evaluated in terms of viability, functionality and morphologically. Islet viability of the BSA group decreased significantly in 72, 96, and 120 h ($P < 0.05$) compared to FBS and ITS groups. While insulin secretion of the BSA group was lower than the others at only 48 and 72 h, the stimulation index was at rational reasonable for transplantation in all groups at 48h and only in the FBS and ITS groups at 72 h. However, islet cells adhere to each other in the FBS group after 48h. Moreover, total oxidant status was the highest and total antioxidant status was the lowest in the BSA group at 120 h. It was concluded that ITS premix could be preferred to FBS and BSA in long-term islet cultures.

Keywords: *Bovine serum albumin, fetal bovine serum, islet, insulin-transferrin-sodium selenite premix, viability.*

INTRODUCTION

Basal media such as Roswell Park Memorial Institute Medium (RPMI), Connaught Medical Research Labs (CMRL), Dulbecco's modified Eagle's medium (DMEM) should include several supplements to keep cells alive and their proliferation, migration and differentiation [1]. Fetal bovine serum is the most frequently used supplement that includes several growth factors, proteins, hormones, trace elements, attachment and spreading factors, etc. Also, it can have some undesirable effects such as contamination risk gathered from unborn calves, and their ingredients may vary depending on the season [1]. Insulin-transferrin-sodium selenite premix is one of the most preferable supplements for serum-free media. Insulin is essential for glucose transport into cultured islets and can act in the growth-stimulatory peptides and somatomedins, transferrin protein that is responsible for cellular iron transport. Sodium selenite serves as a cofactor of sodium selenite-dependent enzymes, catalyzing glutathione metabolism and turnover [2]. Bovine serum albumin is frequently used as a lipid transporter and a protease inhibitor as a

protector and enabling viability in islets. It is also derived from animals and may either be contaminated or contain some undesired substances [3].

In the human, pancreatic islet cells are incubated with CMRL1066 medium (5.6-mM glucose) and 0.5% Human Serum Albumin [4], transfer to the recipient after isolation. In standard conditions, rodent pancreatic islet cells, that are incubated with 10% FBS, transfer to the recipient after 48 h of isolation. Islets need this period to get rid of negative effects of isolation and to get back to normal metabolism. Prolonging the incubation period, decreased the viability and functionality of islet cells, and they adhere to each other. The extension of this period by protecting the viability and functionality of the islets may save the time of both the recipient and the operation teams. Some experimental studies were conducted to increase islet viability and functionality before transplantation such as usage of microfabricated scaffolds [5] or perform co-culture with luteal cells [6]. Also, it was reported that islets embedded in a collagen gel matrix can survive, and their function can preserve more than 8 weeks [7] which was an alternative for prolonging the islet cell viability. Boyuk et al [6] showed that luteal cell co-culture prevents the sticking of islets each other for 96 h by using ITS.

Oxidative stress increases apoptotic cell death and disturbs cell growth and secretion [8]. In *in-vitro* systems, cells are exposed to more oxygen compared than they encounter in the body. Antioxidant mechanism in the cellular ambiance is an important mechanism to keep cell alive and functional [9].

Since the viability and insulin secretion of islet cells begin to decrease after isolation, islet cells should transplant to the recipient as soon as possible. However, if a longer time is needed before implantation, for example, the recipients require a longer pre-transplantation period to complete the immunosuppressant therapy or if the operation team needs more time for transplantation, lengthening the incubation period by preserving viability and functionality of islets could be useful. Therefore, the first aim of this research was to prolong of the islet survival, and to preserve post-isolation functionality by culturing with different supplements (0.3% BSA, 10% FBS and 1% ITS premix) and to reveal which supplement is more beneficial in this regard. Although the effects of individual or different doses of some supplements have been studied so far, there is no study that has yet compared these culture conditions in the literature for a long-time culture and, about the effects of these supplements on oxidative and antioxidative status. Also, the second purpose of this study was to find out the effects of these supplements on total oxidant status (TOS) and total antioxidant status (TAS) of the islet cells.

MATERIALS AND METHODS

Animal procedures and ethical approval

Rats were cared as recommended in the Guide for the Care and Use of Laboratory Animals and the experiments were performed in accordance with protocols approved by the Kirikkale University Local Ethics Committee of Laboratory Animals (2016/48). Islet cells were isolated from 3-month-old, male Sprague-Dawley rats weighing 190-260 g. All groups were studied in triplicate and the trial was repeated 3 times.

Extraction, purification and culturing of the pancreatic islet cells

Chemicals were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) unless otherwise stated. After each pancreas exposed to collagenase type V to obtain islet cells, they were purified with Ficoll 1.100 and Ficoll 1.077 [6].

Three replicates for FBS, BSA and ITS supplement groups were set up for 48, 72, 96, and 120 h. 10% FBS or 0.3% BSA or 1% ITS was supplemented to RPMI 1640 medium with 1% L-glutamine, 1% penicillin-streptomycin-amphotericin B (Lonza), and 25 mM HEPES. A hundred islet cells were seeded in each well. Then, islets were incubated for 120 h.

Islet viability and MTT test

The percentage of viability was calculated for 20 islets as fresh islets and at 48, 72, 96, 120 h. For this purpose, islets were stained with 0.46 μM fluorescein diacetate (FDA) and 14.34 μM propidium iodide (PI) dye solution and were analyzed within 30 minutes [10] using Mat lab [11].

To confirm the cell viability, MTT assay also performed with 50 islets of each group (Thermofischer,13154).

Stimulation index

Fifteen islets were incubated in a CO_2 incubator for 60 minutes at 37°C in 24-well plates in 3.3 mmol/L low-glucose and 16.7 mM high-glucose medium for equalization. The insulin stimulation index was calculated as follows: (insulin content in high-glucose medium) / (insulin content in low-glucose medium [6].

ELISA analysis

Analysis of insulin was carried out using both culture and glucose stimulated insulin secretion supernatant samples that were collected at each incubation time (Rat/Mouse insulin ELISA, cat. Number: EZRMI 13K; Millipore).

TAS and TOS assays

TAS and TOS assays performed with the collected media by the commercial assay kits (Rel-Assay, Turkey) as described by Togar et al [12] and Erel [13] Since the TOS assay is calibrated with hydrogen peroxide and the results were determined in micromolar hydrogen peroxide equivalent per liter (μmol H_2O_2 equivalent/L). Besides, total antioxidant status (TAS) was expressed as mmol Trolox equivalent/L.

Statistics

Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA) for Windows. All data were evaluated using repeated measures analysis of variance with Tukey's post-hoc test to compare the means of each series of experiments. The results are presented as means ± standard errors, and differences with P values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Islet cell viability analysis

Islet cell viability was analyzed by FDA/PI staining and MTT viability assay. As shown in table 1, islet cell viability in the BSA group at 120 h was lower than 48, 72, and 96 h (P=0.0091, 0.0059, 0.0051 respectively) with staining FDA/PI. Also, there was a reduction in all BSA groups at 48, 72, 96, and 120 h compared to the FBS (p=0.0377, 0.0090, 0.0079, 0.0018 respectively) and ITS (0.0065, 0.0072, 0.0053, 0.0023 respectively) groups.

Table 1. *Viability of the islets by staining with FDA/PI in different supplements.*

Incubation times (h)	**Viability (%)**		
0 h	83.68±5.18		
	10% FBS	**1% ITS**	**0.3% BSA**
48 h	86.42±0.31x	88.20±0.70x	75.57±1.95ay
72 h	83.10±.2.20x	82.20±2.59x	62.37±2.91by
96 h	84.15±1.49x	83.88±1.47x	60.23±6.16by
120 h	78.80±1.86x	80.42±1.95x	31.25±5.11cy

Viability of the islets by staining with FDA/PI in different supplements.
a, b: Values with different letters among the same treatment groups were significantly different.
x, y: Values with different letters among the same incubation times were significantly different.

In parallel with the results of the cell viability assay with FDA/PI, a cell proliferation test with MTT assay showed that islets have the lowest viability in the 0.3% BSA group at 120 h compared to at 48, 72, and 96 h (p=0.0098, 0.0059, 0.0255 respectively). FBS and ITS groups' viability was higher than the BSA group at 72, 96, and 120 h (p=0.0059, 0.0371 for 72h and 0.0090, 0.0068 for 96h and 0.00667, 0.00751 for 120h respectively) (Figure 1).

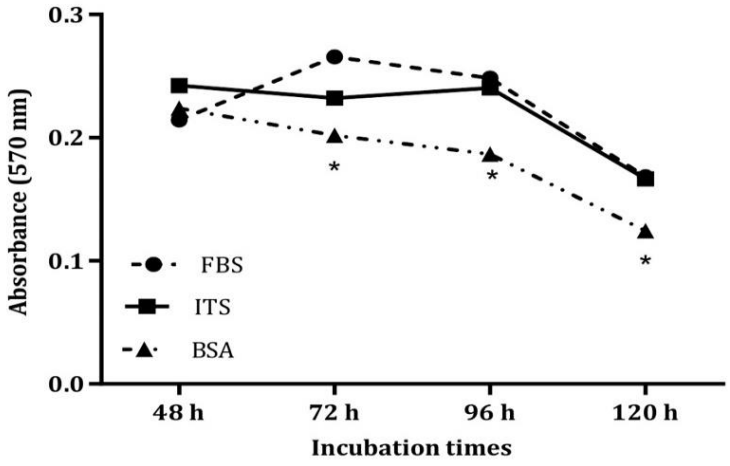

Fig. 1. *MTT assay of islet cells incubated with 10% FBS, 1% ITS and 0.3% BSA*

Total Insulin Level

When total insulin level was compared at 48 and 72 h incubation, BSA groups were lower than the FBS and ITS groups (P<0.0001). At 96 and 120 h, insulin level was not statistically significant in FBS, ITS and BSA groups (Figure 2). Insulin levels secreted by islet cells at 72 h in FBS and ITS groups were higher than those of 96 and 120 h (both p=0.0029).

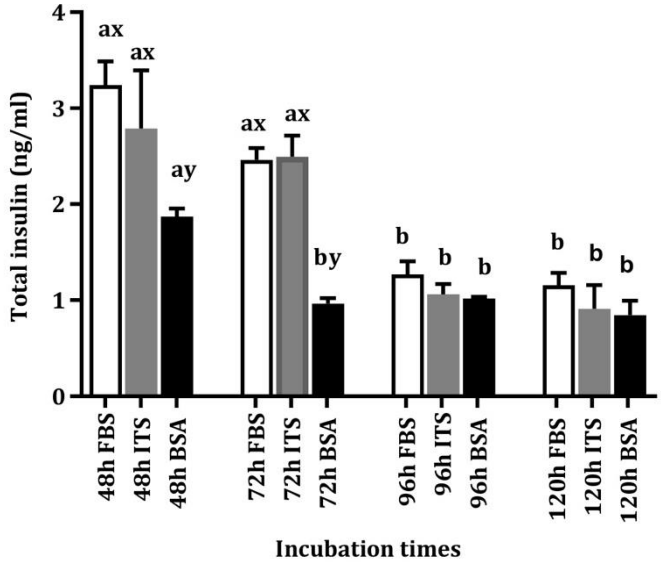

Fig. 2. *Total insulin levels of islets incubated with 10% FBS, 1% ITS and 0.3% BSA (ng/ml). a, b: Different letters shows the differences among the different incubation times of the same treatment groups. x, y: Different letters shows the differences among the different treatment groups in the same incubation time.*

Stimulation index

The stimulation index is used as a parameter that assesses the insulin-releasing function of isolated islets**.** Calculated SI values are shown in figure 3. The SI in the FBS group at 48 and 72 h was higher than at 96 and 120 h (p=0.0360, 0.0347 for 48 h, 0.0544, 0.0169 for 72 h respectively). Among the different incubation times in ITS groups, the SI was the highest level at 48 h (p=0.0081, 0.0189 and 0.0348 for 72, 96 and 120h respectively). The SI of the BSA groups gradually decreased until 120 h (p=0.0589, 0.0407, 0.0356 for 48 h, 72 h and 96 h respectively). While the SI in the FBS group was the highest at 72 h compared to the ITS and BSA groups (p=0.0154, 0.00857 respectively), the SI of the FBS and ITS groups were higher than the BSA group at 96 h and 120 h (p=0.0132, 0.0296 for 96h, p<0.0001, p=0.0002 for 120h respectively). Also, the SI at 120 h was gradually decreased in FBS, ITS and BSA groups respectively.

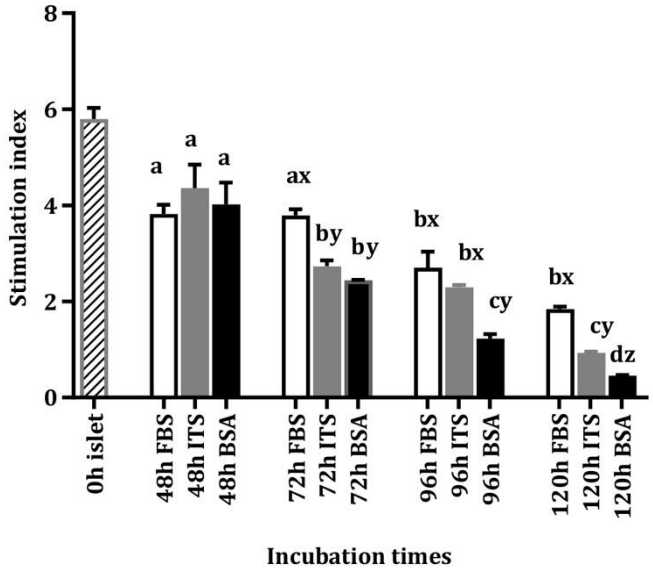

Fig. 3. *Stimulation index of islets. .a, b, c, d: Different letters shows the differences among the different incubation times of the same treatment groups. x, y, z: Different letters shows the differences among the different treatment groups in the same incubation time.*

Microscopic examination of the islet cells

All the incubated cells were examined under the inverted microscope at 48 h (4A-C), 72 h (4D-F), and 120 h (4G-I). When islet cells were cultured with FBS, islets adhered to each other at 72 h (Figure 4D), then adherence increased by consisting of collaterals until 120 h (Figure 4G). The islets did not show any adhesion after 48 h (Figure 4B, 4C), 72 h (Figure 4E, 4F) or 120 h (Figure 4H, 4I) in culture medium containing ITS and BSA respectively. However, the decrease in the viability of the islets with time was also noticed microscopically in the culture medium containing BSA. Cell death was observed in the interior of the islets clearly at 72 and 120 h at BSA groups (Figure 4F, 4I).

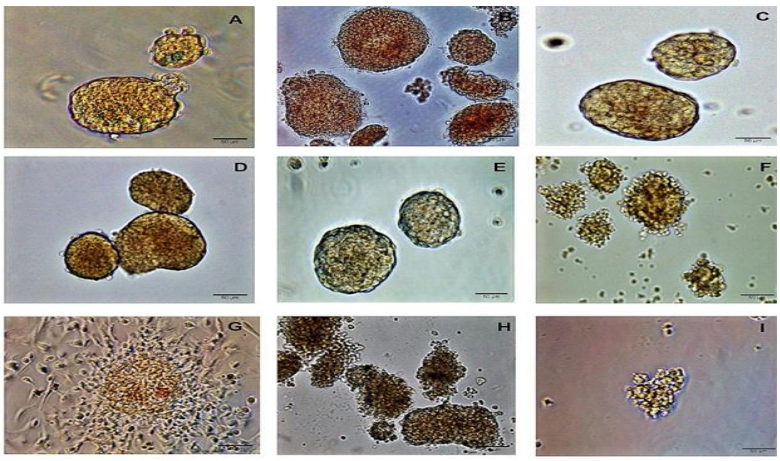

Fig. 4. *Microscopic examination of the islet cells incubated with 10% FBS (A, D, G), 1% ITS (B, E, H) and 0.3% BSA (C, F, I) at 48, 72 and 120 h respectively (×200). Scale bar: 50 µm.*

The total oxidant and antioxidant status of the islets

As shown in figure 5, TOS increased at 96 and 120 h compared to the 48 h and 72 h in FBS (p=0.0581, 0.0563 for 96 h; 0.0248, 0.0483 for 120 h respectively) and ITS groups (p=0.0583, 0.0346 for 96 h; 0.0363, 0.0346 for 120 h respectively). In BSA groups, TOS increased gradually until 120 h. Moreover, after 120 h, TOS was the highest in the BSA group as compared to FBS (p=0.0432) and ITS (p=0.025) groups.
At 96 and 120 h, TAS of the FBS group was lower than those at 48 and 72 h (p=0.0504, 0.0402 for 96 h; 0.0402, 0.0114 for 120 h respectively) (Figure 6). Although, no significance was observed at 48, 72, and 96 h among FBS, ITS and BSA groups, there was a decline in the TAS of the BSA group only at 120 h (P<0.001). Also, TAS of BSA group gradually decreased as the incubation period was lengthened.

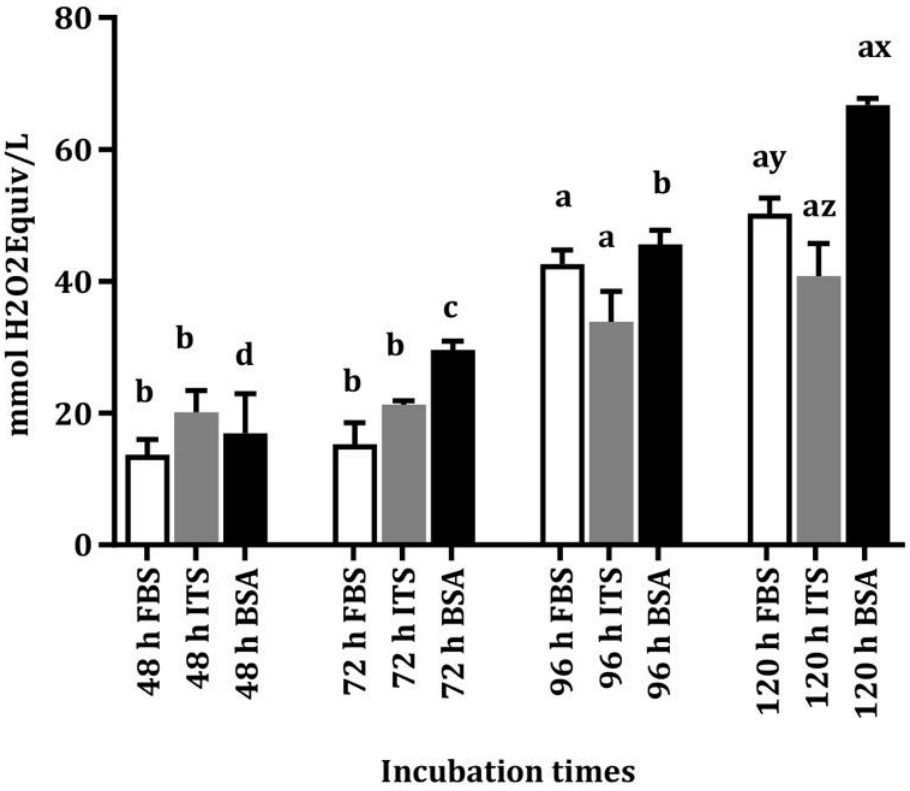

Fig. 5. *TOS of the islets incubate with 10% FBS, 1% ITS and 0.3% BSA in different incubation times. a, b, c: Different letters shows the differences among the different incubation times of the same treatment groups. x, y: Different letters shows the differences among the different treatment groups in the same incubation time.*

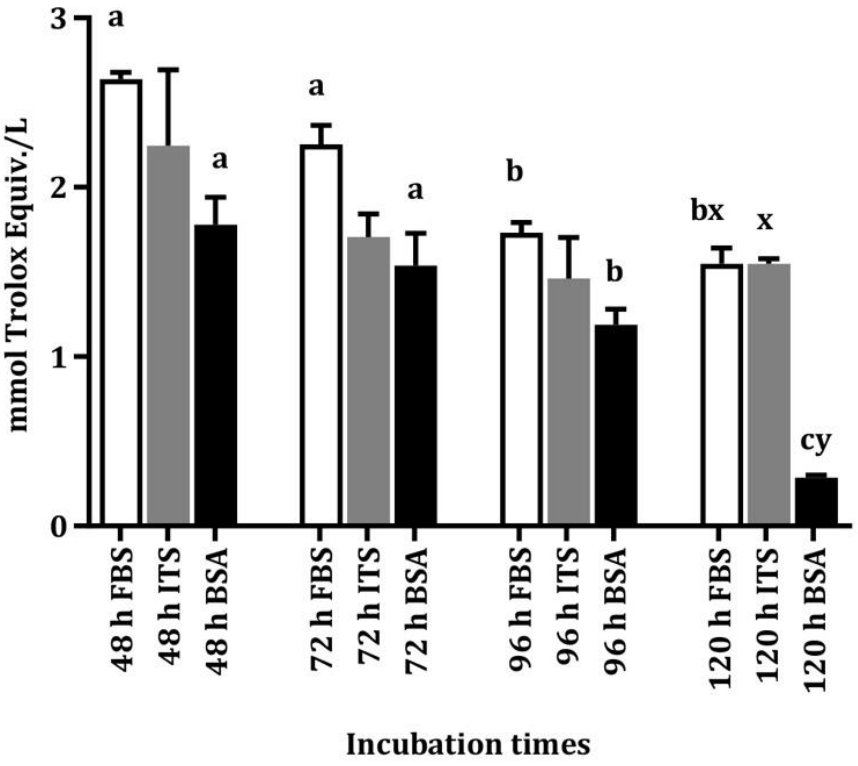

Fig. 6. *TAS of the islets incubates with 10% FBS, 1% ITS and 0.3% BSA in different incubation times. a, b, c, d: Different letters shows the differences among the different incubation times of the same treatment groups. x, y: Different letters shows the differences among the different treatment groups in the same incubation time*

Pancreatic islet cell isolation and transplantation are gaining popularity for patients with type I diabetes. It provides to restore the insulin secretion and blood glucose stabilization. Islets with viability rate of 80% or higher are accepted for transplantation [14]. Moreover, islet cell functionality is depending on islet viability. That's why preserve of islet viability and functionality is important for the success of transplantation during this pre-transplantation period. Also, prolonged this period could be useful when needed.

Cells need some media supplements for their metabolism in culture [15]. Although FBS and FCS are universal growth supplements, because of the disadvantages regarding non-defined ingredients and serious ethical concerns related to increasing numbers of fetuses harvested. There have been new approaches to find alternatives. Rush et al. [16] demonstrated that human islets cultured in Memphis serum-free media preserved *in vivo* and *in vitro* functions. Similarly, Rastellini et al. [17] showed that islet viability rates were 67% and 56% at 60 and 120 days, respectively in culture by using a pyruvate-rich medium. Because the chemical composition of the serum-free medium is defined well, their usage has been increasing. Also, lack of the attachment and spreading factors in serum-free medium formulations are one of their most important advantages [18].

Clark and Chick [19] reported that 5% FBS provided greater insulin secretion than the defined medium with 0.1% human serum albumin up to 7 days. However, Avgoustiniatos et al. [20] declared that islet viability in 10% FBS supplemented medium was higher than that of the medium with 5% human serum albumin. According to Fraga et al. [21], rat

dermal fibroblast media with (2.5 μg/ml ITS or 2.5 ng/ml somatostatin or with 0.5 mg/ml linoleic acid-BSA or 250 ng/ml thyrocalcitonin) can grow better a human B-cell line that was derived from immortalized human pancreatic β-cells, than the medium containing 1% FCS. Also, it was showed that the islet recovery rate and SI were higher in CMRL media with 1% ITS + premix supplement than with 10% FBS supplemented media over 2 months [22]. Gaber et al. [23] reported that islets cultured in a macrophage serum free medium (containing 1% ITS, 1% 1-glutamine, 1% antibiotic antimycotic, and 16.8 μM / L zinc sulfate solution) maintained viability *in vitro* up to 2 months and some samples could be used for transplantation. In this study, according to the FDA/PI staining results, the viability of the islets was more than 80% in FBS and ITS groups at 48, 72, and 96 h and in the ITS group only at 120 h. Although the viability of the FBS group at 120 h decreased slightly under 80%, it was acceptable for transplantation. However, the highest viability rate of the FBS and ITS groups was at 48 h. Similar to the studies as mentioned above [19, 20, 21] both the addition of FBS or serum free supplement increased the viability of islet cells in this study. In our study, it was observed that the viability of islets, cultured with FBS or ITS supplemented medium, was higher in both groups, and there was no statistical difference between them.

Differences in the chemical composition of the serum can have different consequences. Ling [24] showed that 1% of the BSA supplementation to the basal medium increased the islet viability to 75% to 9 days. Also, Behboo [25] has reported 70% of the islet can survive for 30-day cultures in medium with human serum albumin (1.25 mg/mL). Bertera et al. [26] reported that the addition of 0.2% BSA to the isolation media resulted in a 30% increase in islet yield and also increased viability compared to the control group. In this study, islet viability rate (62.37±2.91%) of the BSA groups was not acceptable for transplantation and it was lower than the other treatment groups as prolonged incubation time (72 h and over). Decreasing the viability among BSA groups was statistically significant, especially at 120 h. Bovine serum albumin is probably known as a protease inhibitor that maintains the function and viability in islets. The above-mentioned studies [24, 25, 26] compared BSA activity to the control group, they did not use any other supplements. Because of the viability rate should be at least 80% for transplantation of islets, their viability rate was not within acceptable limits in those studies. Also, 0.3% BSA was incapable of the islet maintenance in our studies. Since BSA is derived from animals, ingredients were not well defined and may differ from each other [3].

Cell viability is also determined by MTT assay and results were supported FDA/PI viability test in this study. The only differences between FDA/PI staining and MTT assay were seen at 48h; while there was no difference in viability among the treatment groups on MTT assay, it was seen that viability of the BSA group was lower than the other groups with fluorescein staining. Also, although the viability of the BSA group was similar at 48, 72, and 96 h on MTT assay, it was lower at 72 and 96 h than at 48 h with FDA/PI staining. Even so, these discrepancies did not affect the fact of the 0.3% BSA had a negative effect on viability.

Islet cells secrete insulin, this ability is important for the success of islet transplantation. In this study, we protected islet viability and functionality by extending the incubation period and adding different supplements. Rastellini et al. [17] showed that tonic stimulation of insulin secretion during the 72-hour culture period of mouse beta cells demonstrated a 20% reduction in the total number of granules. Parallel with viability, insulin secretion by islet cells was higher in the FBS and ITS groups than the BSA groups at 48 and 72 h incubation. There were no significance differences in insulin levels of the

FBS or ITS groups at 48 and 72 h. The point that attracts attention is although their insulin levels decreased at 96 and 120 h, the viability of these groups seemed still high at the same incubation periods. Islet cells began to decompose after 96 hours of culture, especially after culture with BSA. This phenomena reduced insulin secretion of the islets. This decrement might be due to the degranulation in the islets.

In this study, adult isolated rat islets cultured with FBS, ITS and BSA were exposed to low (3.3 mM) and high (16.7 mM) glucose concentrations.

Islet functionality is measured by the SI. In the current study, while the SI was the highest in ITS and BSA group at 48 h among the incubation periods (72, 96 and 120 h), SI of the FBS group was higher at 48 and 72 h than at 96 and 120 h. According to [27], SI of three or more is suitable for transplantation of islets. Thus, the SI of the FBS, ITS and BSA groups at 48 h (3.82, 4.36, 4.02 respectively) and the SI of FBS at 72 h (3.79) may be enough for transplantation. Also, it is thought that since viability and insulin release was sufficient, the stimulation index of ITS (2.734) can acceptable at 72 h. Carter et al [28] declared that Miami and hCell media, that include glutathione as a supplement, provided better SI and insulin release from the islets. Also, Fraga et al. [22] reported that CMRL media with 1% ITS + premix supplement effects SI positively. Murdoch et al. [29] showed that the preservation of human cultured islets may be affected by media supplements too. One of these components may support islet metabolism by having antioxidant and antiapoptotic properties and enhance the islet viability and function.

The most important problem in long-term culture is the adhesion of the islet to the well when FBS is used as a supplement. Since morphological and physiological changes occur in the adherent cell, their function is impaired. Therefore, we used ITS in a previous luteal cell and islet cell coculture study to prevent them sticking to each other [6]. In this study, although islet adhesion began after 48 h in the FBS group, ITS treatment helps the islets maintain the vitality and long-term culture conditions by anti-adhesion effect. Bertera et al. (2012) is reported that 0.2% BSA was preferred to FBS in terms of anti-adhesion effect until 24 h of incubation. On the contrary to 0.2% BSA, 0.3% BSA failed to prevent islet death after 48 h in this study. As a result, it can be considered that BSA was insufficient to prevent islets from adhering in prolonged incubation times. Also, differences in the effects of BSA on adhesion with Bertera et al [26] may be caused by variations of the serum ingredients.

Oxidative stress is related to the release of free radicals, especially reactive oxygen species (ROS). Islets are sensitive to free radicals [30]. In this study, as an indicator of the ROS, TOS gradually increased as the incubation period extended to 120 h with FBS, ITS and BSA supplementation to the medium. Also, since the BSA group has the higher TOS compared to the FBS and ITS group at 120 h incubation. It is thought that BSA may not protect the islets against oxidation that is derived from prolonged culture. The reason for this condition might be quite a high rate of islet death.

Treatment of islet preparations with potent antioxidants may reduce oxidative stress [31]. Fetal bovine serum is composed of hormonal factors stimulating cell growth, transport proteins carrying hormones, minerals and trace elements (e.g. transferrin) with some having antioxidant activity, attachment and spreading factors [2]. Because of the supplementation of glutathione (GSH) could decrease apoptosis and reduce intracellular ROS during islet isolation [31]. We used also ITS, which includes sodium selenite that serves as a cofactor of sodium selenite-dependent enzymes, catalyzing glutathione metabolism and turnover. As shown in Figure 6, while the FBS group has higher antioxidant activity at 48 and 72 h than at 96 and 120 h, the antioxidant effect of ITS not

differs among the different incubation periods. However, except at 120 h, islets incubated with FBS, ITS and BSA supplemented media had no statistical differences in terms of antioxidative effects at the same incubation times; at 120 h, the antioxidant activity of the BSA was dramatically decreased. Probably, although albumin in BSA is an antioxidant, its content may time-dependently decrease. Similarly, Faure et al. [32] demonstrated that HeLa cells with 20 g/l BSA were incubated at 37 °C for 24 h, and their antioxidant capacity was decreased because of the loss of albumin.

This study indicated that BSA (0.3%) was not enough to improve islet function in vitro. FBS and ITS, that is one of the serum free media supplements, helped maintain the islets viability and insulin secretion until 72 h, and, they have high antioxidant activity. Although it seemed that the FBS group has a more positive effect than the ITS group in terms of viability, functionality, and antioxidant activity, FBS caused adhesion in culture at 72 h and thereafter, which was undesired for transplantation. In addition, incubation of the islet cell in serum-free media has many advantages: decrease in the usage of the annual numbers of fetuses (reduction), it may replace the fetal serum and it may improve animal welfare. Serum-free media improves animal welfare, and it helps achieve the 3R rule (Replacement, Reduction and Refinement). Since ingredients of the ITS premix are defined very well and can standardized, usage of it may more reliable and effective for islet cell incubation up to 72 h.

CONCLUSION

The islet cell culture should be uniformed for optimized islet isolation and transplantation. Because of the range of islet sizes is similar in rats [33] and humans [34] (50-500 μm and 50-250 μm respectively) and beta cells make up rates are 70–80% and 50–70% of cells in islets in rat [34] and humans respectively; we thought that our findings can use for improving human islet cell culture. For this reason, studies for media optimization of islet culture will take a significant role in the performance of oncoming studies of human islet cell transplantation. In transplantation applications, extending the duration of islet cell culture after isolation may allow time to increase the viability and functionality of islets. In addition, long-term culture also provide time to prolong islet viability when selecting suitable islet donors matched to the recipient. However, in future studies, the results should be evaluated in terms of insulin major gene transcription factors.

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Conflict of Interest. "The authors declared that there is no conflict of interest."

Authorship Contributions. Concept: G.B., A.A.Y., Design: A.A.Y., G.B, Data Collection or Processing: G.B., A.A.Y., Analysis or Interpretation: A.A.Y., G.B., Literature Search: A.A.Y., G.B., Writing: A.A.Y., G.B., Reading and review: G.B., A.A.Y.

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