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Original Research

The synergistic effect of ω3 and Vit D3 on glycemia and TNF-α in islet transplantation

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Abstract: The current treatment of type 1 diabetes consists of insulin administration. Transplantation of islets of Langerhans is considered very favorable because the full effect of insulin treatment cannot be obtained in severe cases. Although agents such as omega-3 (ω3) and vitamin D3 (Vit D3) are known to contribute to the success of islet allo-transplantation (ITX), in this study we aimed to experimentally determine their effects on glycemia and TNF-α production. Wistar albino rats, which were used as recipients, were given ω3, Vit D3, and islets by gavage, and intraperitoneal- and intraportal injections, respectively. Daclizumab (DAC) was used for immunosuppression. Glycemia levels decreased in rats treated with ω3 and vit D3. TNF-α increased in all groups due to application of STZ. After ITX (day +1), the weakest increase was observed in the ω3 + Vit D3 group. In the ITX+DAC group, compared with that of ITX only, DAC was shown to decrease levels of TNF- α following ITX, only in control group, however, similar levels of TNF-α were observed in other groups. The values in the treated groups were already lower than those of the controls in the ITX group and also remained almost equal in the ITX+DAC group. We suggest that the use of ω3 and Vit D3 together will improve the pro-inflammatory aspect encountered during and after ITXs, and contribute to the reduction of the dose of immunosuppressants in these procedures.

Key words: ω3, Vit D3, islet transplantation, glycemia, TNF-α.

Introduction

Insulin therapy, dietary restrictions and physical activity are methods that are currently used for the treatment of type 1 diabetes (T1D) (1). Exogenous insulin forms are unable to replace the nictomeral rhythms of the natural hormone (2). In some patients with T1D, intensive insulin therapy can not compensate the glycemic imbalance (3). In addition, the risk of hypoglycemic episodes increases if insulin is injected in multiple doses per day. Episodes of hyper-and hypoglycemia are inevitable in some diabetics who are referred to as "brittle", those who are also under high risk of developing diabetic complications. Thus, alternative methods have been introduced in the field of investigation to improve glucose control (4). Normal islets produce the right amount of insulin because they are physiological. Precisely for this property, they can be used in transplantation to obtain a better and continuous control of glycemia and reduce the risk of complications from diabetes mellitus (DM) (5). Although insulin independence is found in a small percentage of patients five years after transplantation, its importance can be seen in the maintainance of glucose homeostasis (6). Transplantation of pancreatic islets is performed to increase the quality of life of patients (7, 8). It has been shown that diabetes complications such as microvascular disease, neuropathy, retinopathy and chronic renal failure can be prevented at least in part by transplantation of islet cells (7, 9).

Clinical use of islets in the treatment of T1D was admitted as a biologic product and a drug product by the Food and Drug Administration from the point of view of regulatory status (10). However, recurrence of autoimmune diabetes could contribute, even if partially, to destruction of islet grafts in type 1 diabetics.

Omega-3 (ω3) fatty acids are known to be beneficial for human health (11, 12) and were shown to be useful in preventing and improving autoimmune disorders (13). The risk of type 1 diabetes and islet autoimmunity is reduced if fish oil is taken from 1 year of age (14-16).

Flax belongs to the family Linaceae and is available in the world market as a functional food. It contains the essential ω3 fatty acid, alpha-linolenic acid (ALA), which may inhibit the formation of pro-inflammatory cytokines (17, 18). Tumor necrosis factor-α (TNF-α) is one of the cytokines involved in inflammation, and its decrease in mononuclear cells was observed in 27% of healthy men fed with a diet containing flax for four

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weeks (19). It was shown that the combination of 14-epi-1, 25 - (OH) 2D3-analog (TX 527), and IFN-β delays the recurrence of autoimmune diabetes in transplants of islets in NOD mice (20). It was demonstrated that 1,25(OH)2D3 could protect intraportally transplanted islets against nonspecific inflammation and improve islet graft survival (21). In our study, along with blood sugar that would act on the islet transplant survival, we also aimed to immunologically investigate a peri-transplant value using TNF-α as a parameter.We examined the effect of ω3 both alone and in combination with vitamin D3 (Vit D3) on the survival of graft after transplantation of islets of Langerhans (ITX) looking from an immunologic point of view, but mostly on immunosuppression dose. We did this considering the relationship between the decrease of this cytokine and T1D, and that the positive effect of ω3 on T1D has been demonstrated in experimental animals.

Materials and Methods

Experimental design

The rats were obtained from the Department of Laboratory Animals Science of the DETAE. We divided them into two groups as recipients and donors, and kept them in a 12-h light and 12-h dark environment with free access to standard rodent chow and tap water. The recipients were given Hank's balanced salt solution (HBSS), streptozotocin (STZ) dissolved in 0.9% saline solution, pancretic islets, ω3, Vit D3, and daclizumab accordingly to their respective groups. All experiments were approved by the Local Ethics Committee for Animal Use at the Istanbul University.

Diabetes induction in the recipients

Animals in the diabetic group were made diabetic with STZ (Sigma, USA) (60 mg/kg) by intraperitoneal injection and waited at least 15 days before any intervention. Thus, the 15th day was taken as day 0 in the case of ITX. Recipients whose nonfasting blood glucose levels after 24 hours were at least 200 mg/dL were considered diabetic.

ω3 and/or Vit D3 treatment

ω3{7mg/kg, ω3 "700 mg" Solgar Vit. Ltd. USA [each capsule contained 1,5 gr total fat, -3 polyunsaturates providing eicosapentaenoic acid (EPA) 360 mg, docosahexaenoic acid (DHA) 240 mg, other fatty acids 100 mg, Vit E (d-alpha tocopherol) 4IU]} was administered or Vit D3 (5μg/kg, Alpha D3 1μg, 50 capsules, 1α-Hydroxyvitamin D3, Teva Pharmaceutical Industries Ltd., Israel) or ω3 + Vit D3. The sera for evaluation of TNF-α were stored. The administration of ω3 and Vit D3 was through gavage (in 200 μL coconut oil) and intraperitoneally (in 1 mL peanut oil), respectively.

Immunosuppression

Application of daclizumab (Zenapax, Roche) (10 μg/mL of sterile water) for immunosuppression was performed intravenously administering in a dose of 0.05 mg/kg body weight (22, 23) before ITX (day 0) and on days +1 and +2 *(days 15, 16 and 17 in the experiments without ITX, respectively).*

Glycemia

Glycemia values were obtained from tail vein blood using a glucometer (Accu-Chek Active, Roche).

TNF-α

In all groups the sera were taken to measure TNF-α [TNF-alpha (Rat) ELISA Kit, Biosource] levels using enzyme-linked immunosorbent assay (ELISA). Measurements were made before STZ treatment, before transplantation (day 0), and on days +1, +2.

Recipients

Ninety six syngeneic Wistar albino rats (male, aged 16-18 weeks, weighing 220–250 g) were obtained from the Department of Laboratory Animals Science, DETAE, Istanbul University. Rats were divided into four main groups {Group 1 [Hank's balanced salt solution (HBSS) treated]; Group 2 [Streptozotocin (STZ) treated]; Group 3 [STZ + islet transplantation (ITX) treated]; Group 4 [STZ + ITX + daclizumab (DAC) treated] subdivided in four groups each [n=24: control (n=6), ω3 treated (n=6), Vit D3 treated (n=6), ω3+Vit D3 treated (n=6)] (Table 1 and Figure 1).

Donors

Three Wistar albino rats (aged 16-18 weeks, weighing 220-250 g) for each recipient were used as donors.

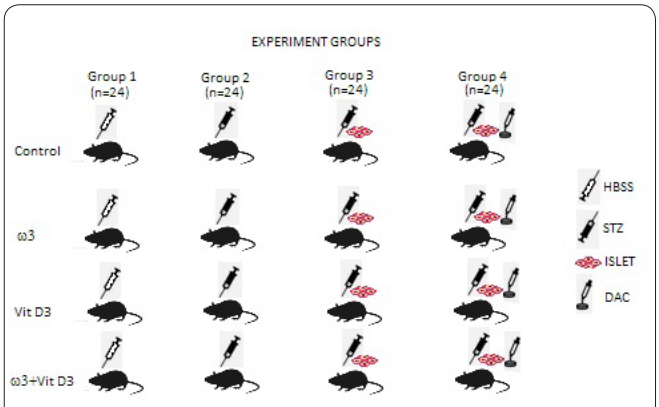

Figure 1. Groups of recipients.

Table 1. Wistar albino rats divided into four main groups subdivided in four groups each.

	GROUP 1	GROUP 2	GROUP 3	GROUP 4
	HBSS (N=24)	STZ (N=24)	STZ + ITX (N=24)	STZ + ITX + DAC (N=24)
SUBGROUPS	CONTROL	CONTROL	CONTROL	CONTROL
	ω3	ω3	ω3	ω3
	VIT D3	VIT D3	VIT D3	VIT D3
	ω3 + VIT D3	ω3 + VIT D3	ω3 + VIT D3	ω3 + VIT D3

Islet isolation

The islet isolations were performed using a mechanical/enzymatic method (24).

Surgical procedure and disruption of the pancreas

A laparotomy was performed and the xiphoid process was cut. The head of the animal was turned to stand closer to the operator. The duodenum, which is located about 2 cm from the stomach, was distended in a latero-distal direction in order to bring out the common bile duct, and the area that opens to the duodenum was clamped. The liver was advanced into the rib cage. A loose knot was prepared in the proximal part of the common bile duct. After making a small incision in the common bile duct, a cannula (16 G) was introduced to dispense HBSS and the knot was tightened to ensure stability. The pancreas was distended with 10 mL of HBSS, dissected from the neighboring organs, and disrupted mechanically in a test tube containing HBSS to maintain vitality of the tissue. The pancreatic tissue was cut with scissors for about 5 minutes until it separated into small pieces, and artifact fat was removed using a Pasteur pipette.

Digestion phase

The pancreas pieces were transferred into a glass tube and the excess HBSS was removed. For digestion, 4mg of Collagenase P (Boehringer Mannheim, Germany) / pancreas was used. The pancreas was incubated for 10 minutes at 37°C and simultaneously oxygenated with 95% O_2 + 5% CO_2. Homogenated pancreatic tissue fragments were put into a large beaker and digestion was stopped with cold HBSS and the content stirred with a plastic pipette.

Washing phase

The HBSS of the supernatant was removed after waiting for about 3 minutes to remove the collagenase. HBSS was added again, and this washing process was repeated 3-4 times.

Collecting phase

Islets were hand-picked by taking a small amount from the pellet that remained in the beaker under a dissecting microscope.

Islet transplantation

For intraportal islet transplantation, the peritoneal cavity of the animal was opened with laparoscopic surgery and with the bowel placed outside on the left abdominal side, the portal vein was displayed. Approximately 1000 hand-picked islets were suspended in 500-1000 µL HBSS and injected into the portal vein using a 24G butterfly catheter. After removal of the catheter, the point of cannulation was manually compressed using a piece of Gelfoam until bleeding is stopped (approximately 5 minutes). The abdominal cavity was closed by surgical suturing at the end of the procedure.

Anesthesia

Pentothal sodium (50 mg/kg i.p.) was used for anesthesia of both recipients and donors.

Statistical Analysis

Values are expressed as mean and SEM. The analysis of inter- and intra-groups were performed using ANOVA and Dunnett's t-test [control (before STZ) and test groups (days 0, +1, and +2)], respectively. The differences between the values of glucose and TNF- from days +1 and +2 after transplantation were evaluated using Student's -t test.

Results

Glycemia

The blood glucose levels of the rats in the HBSS group were normoglycemic and on day 17, the blood glucose levels were measured as 107±9 mg/dL and 116±12 mg/dL, 115±11 mg/dL, 109±12 mg/dL in the controls and the ω3, Vit D3, and ω3 + Vit D3 groups, respectively (Figure 2A).

In all rats of STZ-only group at 15th day of STZ injection, blood glucose levels were measured over 200 mg/dL, which reveals STZ-induced diabetes in rats. The increase of blood glucose levels after the administration of STZ, was measured in ω3 and Vit D3 and ω3 + Vit D3 groups at a lower level than that in the control group 399±13 mg/dL , 368±24 mg/dL, 362±26 mg/dL, 479±15 mg/dL, respectively. Even in measurements taken after days 16 and 17 this difference was maintained and the difference was highly statistically significant in all groups compared with controls (*p <0.001, Figure 2B).

In the rats of the ITX group 15 days after the STZ injection, an increase of 200 mg/dL was observed in blood glucose in a manner similar to the STZ group. On days +1 and +2 after islet transplantation, blood glucose levels fell to normal and the difference between the ω3, Vit D3, ω3+Vit D3 groups and the control group had disappeared (Figure 2C).

In rats of DAC group treated with STZ, the decline of blood sugar was observed on days +1 and +2 after islet transplantation and injection of DAC. However, the decline in normal glucose levels in the control DAC group was scarce on day +1 compared with the ITX group (Figure 3), but the decline was down to the same level in all groups on day +2 (Figure 2D).

TNF-α

TNF-α levels in the HBSS group remained normal, and TNF-α levels of the controls, and ω3, Vit D3 and ω3 + Vit D3 groups were 16.7±0.5 pg/mL, 19.0±1.5 pg/mL, 15.0±1.3 pg/mL and 16.3±1.7 pg/mL, respectively, on day 17 (Figure 3A).

On day 15 after injection of STZ made to induce diabetes in rats of STZ group, levels of TNF-α showed an increase in all groups. Comparing the increases, the levels in controls measured higher compared with the ω3, Vit D3 and ω3 + Vit D3 groups 64.8±9.7 pg/mL, 47.9±3.8 pg/mL, 41.4±3.9 pg/mL, and 37.5±5.6 pg/mL, respectively. While the difference between the ω3 / Vit D3 groups and controls decreased in the measurements taken after days 16 and 17 in the STZ group, the difference between the ω3 + Vit D3 group and the control group was found significantly higher (*p <0.001, Figure 3B).

In rats of ITX group, levels of TNF-α increased in all groups at day 15 after the injection of STZ. After islet

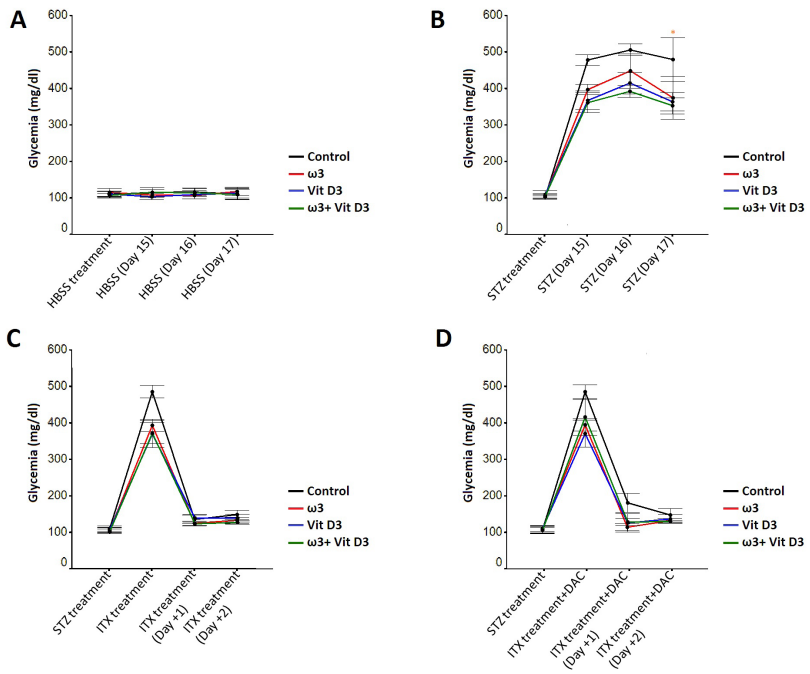

Figure 2. Glycemia values in rats. Glycemia values in rats treated with HBSS on the first day only and ω3, Vit D3, ω3+Vit D3 during the whole experimental period together with controls **(A);** Glycemia values in rats treated with STZ on the first day only and ω3, Vit D3, ω3+Vit D3 during the whole experimental period together with controls (*p<0.001) **(B);** Glycemia values in rats treated with STZ on the first day only and ω3, Vit D3, ω3+Vit D3 during the whole experimental period together with controls. ITX was performed on the 15th day after STZ injection **(C);** Glycemia values in rats treated with STZ on the first day only and ω3, Vit D3, ω3+Vit D3 during the whole experimental period together with controls. ITX was performed on the 15th day after STZ injection; DAC was used for three days **(D).**

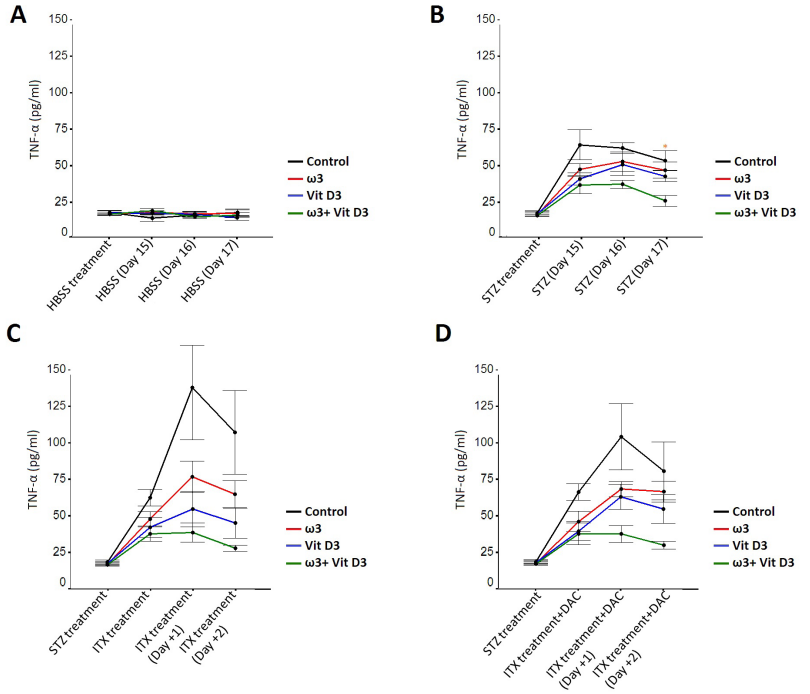

Figure 3. TNF-α values in rats. TNF-α values in rats treated with HBSS on the first day only and ω3, Vit D3, ω3+Vit D3 during the whole experimental period together with controls **(A);** TNF-α values in rats treated with STZ on the first day only and ω3, Vit D3, ω3+Vit D3 during the whole experimental period together with controls (*p<0.001) **(B);** TNF-α values in rats treated with STZ on the first day only and ω3, Vit D3, ω3+Vit D3 during the whole experimental period together with controls. ITX was performed on the 15th day after STZ injection **(C);** TNF-α values in rats treated with STZ on first day only and ω3, Vit D3, ω3+Vit D3 during the whole experimental period together with controls. ITX was performed on the 15th day after STZ injection; DAC was used for three days **(D).**

transplantation (day +1), levels of TNF-α in the control group showed a large increase as 138.1±34.2 pg/mL, at the same time, the groups treated with ω3 and Vit D3 levels of TNF-α were at lower levels, 76.9±10.2 pg/mL and 54.8±11.5 pg/mL, respectively. However, in the ω3 + Vit D3 group, levels of TNF-α at 38.8 ± 6.1 pg/mL were determined at a level close to those seen with administration of STZ. Together with the observed reduction of TNF- α on the day +2 compared with day +1 following islet transplantation, levels of TNF- α in control group were still remained high (Figure 3C).

High levels of TNF- α in the blood due to STZ were

found to be diminished in DAC group both on day +1 (103.8 ± 21.5) and day +2 (80.3 ± 18.9), in comparison with ITX control group (Figure 2C). TNF-α levels were not different in ω3, Vit D3 and ω3 + Vit D3 groups with respect to presence of DAC (Figure 3D).

Discussion

It is widely accepted that high blood sugar leads to a traumatic effect in the endothelium and other tissues with which blood comes in contact, and the physiologic response to tissue trauma results in inflammation (25). The same effects occur in regions of tissues where a transplant is verified.

In our study, increases in blood sugar after application of STZ remained lower in rats treated with ω3 and Vit D3 than in the control group. Similarly, anti-inflammatory effects have been shown with the dietary EPA (25) and in the reduced infiltration of inflammatory cells with 1,25(OH)2D3 treatment (21), in which graft function remained for two weeks and more than two weeks under euglycemic conditions in 50% and 80% of islet recipient control and 1,25(OH)2D3-treated rats, respectively (21). Also, in our study we found that glycemia in the ITX and ITX+DAC groups, which is an important parameter for graft survival, was lower in the ω3, Vit D3 and ω3 + Vit D3 groups compared with controls.

To avoid adverse effects of suppressing the immune system, new therapies aim to modulate autoimmunity in particular. Dendritic cells (DCs) being in proximity of T-cells, may activate them also locally (26-28).

Being professional antigen-presenting cells (APCs), DCs can be involved in allograft rejection and play possible roles in the immunity or tolerance in the context of the immune system (29, 30). They can inhibit alloantigen specific T-cell proliferation and prolong allograft survival in recipients (31, 32). They can induce apoptosis of autoreactive CD4+ T cells and convert naive CD4+ T cells into antigen-specific Treg cells which can in turn convert mature dendritic cells into DCs with a regulatory capacity (33-36).

1,25(OH)2D3 has an immunomodulating capacity by inhibiting autoimmune diseases and allograft rejection (37). It inhibits alloreactive T cell activation by focusing APCs, and in particular DCs (38-42). It inhibits their differentiation, maturation, and activation *in vitro* while promoting their apoptosis (38). EPA is also involved in reduction of their maturation (43). 1,25(OH)2D3 can change the immunogenicity of murine dendritic cells and assume a tolerizing form, which changed T-cell-stimulatory capacity *in vitro* and *in vivo* (32).

DCs that have been exposed to ω3 fatty acids do not migrate to lymph nodes, but remain at the site of inflammation, induce apoptosis in effector T cells that infiltrate the inflammed region, thereby contributing to the resolution of inflammation (44). Resident macrophages in the liver play a role in nonspecific inflammation after islet transplantation. Serum TNF-α and macrophage infiltration into transplanted islets increases after ITX (21). Inhibition of macrophages reduces levels of proinflammatory cytokine TNF-α after transplantation, which leads to improved islet engraftment (21, 45). 1,25(OH)2D3 inhibits the accumulation of macrophages in the transplanted islets' site and serum, and consequently decreases islet graft loss (21).

TNF-α is involved in diabetes development via islet antigen-specific Th17 cells (46, 47), associated with altered immune cell responses, and consequent β-cell destruction (48-51).

It was found that expression of TNF-α was downregulated in serum in 1,25(OH)2D3-treated rats following islet transplantation, which improved islet graft survival (21). We treated the rats with Vit D3 and ω3 either seperately or together for 17 days, 15th day being the transplantation time point. ω3 PUFA derivatives have been shown to play a role in the modulation of macrophage function (52-54), and anti-inflammatory M2 macrophages become relevant while the pro-inflammatory M1 macrophages are lost (12, 55). If somehow Vit D3 produces toxic effects on macrophages (21), to our reasoning, elevation of M2 could be provided by ω3, which would protect the innate immune system.

During organ procurement and islet isolation, islets of Langerhans undergo stress conditions that lead to shortened survival. Hypoxia, inadequate nutrient supply, and instant blood-mediated nonspecific inflammatory response (IBMIR) are the main factors of reduced graft function after transplantation. Islet cells are affected by oxidative injury during isolation and transplantation (51, 56, 57).

The response of TNF-α to the application of STZ, which is a traumatic factor, is mitigated with administration of ω3 and Vit D3, positive effects were obtained by using them together. Our results suggest that administration of ω3, which is an anti-oxidant, and Vit D3 before and after islet transplantation, enhances islet cell function and attenuates cytokine-induced injury in concordance with other studies in which TNF-α was downregulated in serum of 1,25(OH)2D3-treated rats following islet transplantation (21), eupatilin as an antioxidant was found to protect mouse pancreatic beta cells in part by suppressing caspase-3 cleavage (51) , and EPA inhibited caspase 3-cleavage (58).

Although the transplantation of isolated islets of Langerhans is believed to be an attractive curative treatment for diabetes mellitus (51, 59), in our study the response of TNF-α was very restricted as a reaction to transplantation of islets, which constituted a second traumatic application following administration of STZ in the control group. IBMIR was found to affect the outcome of ITX after which serum TNF-α increased (21, 60).

Endothelial dysfunction (ED) is related with early atherosclerosis in T1D (61). Hyperglycemia as an important functional change that leads to oxidative stress in which NO production is impaired (62-64). Due to the decreased availability of NO, vasoconstrictor factors released by the endothelium are the main cause of ED (64), and migration of blood cells into the arterial wall, induces synthesis of inflammatory cytokines, which contribute to atherogenesis (64,65). Diabetes was found to inhibit plaque regression in atherosclerosis and TNF-α significantly reduced in the EPA group compared with control groups (66,67).It was considered that EPA possibly through functional changes in DCs reduced the number of T cells (28,66). In a model of allogeneic islet transplantation, as dendritic cells assumed a tolerogenic profile after treatment with 1,25(OH)2D3,

the latter was consequently considered as an adjuvant tolerogenic treatment factor *in vivo* (32). Thus, as DCs become tolerant, the dose in immunosuppression could be diminished, and additional macrophage activation measured. ω3 may be used in the treatment of non-alcoholic fatty liver disease because effects were shown to be beneficial on lipid metabolism and oxidative stress in the liver (68). It could also be useful in steatohepatitis encountered following ITX.

In our study traumatic impact caused by transplantation of islets was restrained to a minimum when ω3 and Vit D3 were given, especially if given together, and the response of TNF-α in subjects treated with ω3 and Vit D3 together remained similar only to those seen following the application of STZ.

Although the response of TNF-α in the transplantation of islets was suppressed in rats in the control group because of the use of DAC, it did not undergo a change in the group treated with ω3 and Vit D3 that already contributed to maintain cytokines at lower levels. As it is suggested that a long-term reduction in mature DCs, associated with transplantation tolerance, could also be induced by peritransplant administration of other immunosuppressive agents (42, 69), we propose a combination of ω3 + Vit D3 together with immunosuppressants, and suggest realization of studies on effects of DAC family on DCs, as well as DAC selectivity.

1,25(OH)2D3 inhibits alloantigen-specific immune-mediated rejection (70), hence it may have potential therapeutic benefits in extending graft survival in ITX (21). Less calcaemic analogs, especially analog TX527, which was confirmed to be more effective and to have no adverse effects (71, 72), could be preferred or in combination with ω3. Doses of Vit D3 may be reduced because its immunomodulatory activities are seen at supraphysiologic doses. However, a difference has been shown in the antiinflammatory response to Vit D regarding sex steroids (61, 73-75). We used male rats and suggest that comparative studies using both sexes are needed. In addition, if islet transplantations into omental tissue will be realized, differences with sexes must be taken into consideration in the event of Vit D3 supplementation. Moreover, even if in wintertime there is no evidence for requirement of high-dose Vit D3 in prediabetes to improve glucose homeostasis (76), seasonal adaptation in dose must be taken into consideration after ITX.

Co-administration of Vit D3 analogs with other immunomodulating agents augments their disease-modifying abilities (20, 72, 77, 78). Combination immunotherapy with subtherapeutic doses of anti-CD3 mAb, CsA, and TX527, delayed diabetes recurrence after syngeneic islet transplantation (72). Similarly, we suggest the combination of Vit D3 with ω3 in ITX. Although the detailed mechanisms underlying the beneficial effects of ω3 have not been completely defined (12), it has been shown to reduce the incidence of cardiovascular events in humans, and induce substantial regression of atherosclerotic lesions (28, 66, 67). Therefore, on the assumption that ω3 is involved in antagonizing inflammatory cell death (79), we suggest that it could be useful to add EPA to follow-up therapy after ITX. As with the suggestion that reducion of the dietary v6/v3 ratio may protect against v6-rich Western diet-induced steatohepatitis (80), the same opinion could be supported for preventing possible steatohepatitis encountered after intraportal ITX.

In summary, considering the adverse effects of the use of immunosuppressants on immunity, and assuming that the anti-inflammatory effect obtained with the use of ω3 and Vit D3 will contribute to the reduction of the dose of immunosuppressants, we may consider a new perspective for transplants regarding immunosuppression, and new hope for patients undergoing islet transplantation, taking care, however when interpreting experimental results in rats.

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