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Experimental assessment of the diabetogenic potential of bovine enterovirus in Wistar rats

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Abstract

The involvement of enteroviruses in the development of type 1 diabetes (T1D) has been long studied, since they may affect the insulin producing β cells directly or may act in the pathology through the development of autoimmunity. Therefore, this study investigated the effects of exposure of an animal model (Wistar rats) to bovine enterovirus (BEV) through biochemical, histological, molecular and immunological analyses. Male Wistar rats were divided in five groups: control, enterovirus, immunized with oil-adjuvanted BEV-vaccine, immunized and challenged with BEV (immunized+enterovirus) and streptozotocin (STZ) treated. Stools specimens were collected during the experimental period and after, the animals were killed for blood and tissue collection. An increase of blood glucose levels was observed in the immunized, immunized+enterovirus and STZ groups, while morphological alterations in islets and increased anti glutamic acid decarboxylase (GAD) antibodies were only observed in the STZ animals. The presence of viral RNA in stools was ascertained in the groups which ingested the contaminated water. Thus, BEV did not induce clinical diabetes in orally infected rats, but immunization with inactivated BEV lead to blood glucose metabolism impairment.

Key words : bovine enterovirus, diabetes, autoimmunity, viral excretion.

INTRODUCTION

There is a well-documented genetic basis for type 1 diabetes (T1D), however, the rising incidence of the disease, in both developed and developing countries, in recent decades and a reduced contribution of high risk human leukocyte antigen (HLA) genotypes, indicate that non-genetic factors are important [1-3]. Significant evidence has mounted linking enteroviral infections to the initiation of the disease [4] and to progression from islet autoimmunity to overt T1D [5, 6], although some data suggest that viral infections may be protective against autoimmunity (i.e. hygiene hypothesis) [7, 8].

The mechanism which viruses may trigger T1D causing the selective destruction of insulin-producing β-cells, is unclear. However, some hypothesis have been proposed: (1) direct infection of β-cells, where cells are damaged by an acute cytolytic effect and by a slower immune-mediated damage [9]; (2) bystander activation, where viral infection induces tissue damage, with release of sequestered antigens, which activates autoreactive T lymphocytes that were not directly involved in the initial reactivity to the virus [10]; (3) molecular mimicry, where viral proteins mimic the amino acids sequence of autoantigens, stimulating T cells to cross-react with and damage host tissue [11, 12]; and (4) viral persistence [9].

Despite their shortcomings, animal models remain indispensable tools to map pathological mechanisms. So far, most studies were carried out in models of spontaneous diabetes such as the non-obese (NOD) mouse, using the intraperitoneal route of infection. This route causes consistently more morbidity and even mortality than the oral infection route [13]. Nevertheless, it is relevant to study the pathogenesis of T1D comprising the oral route, due to its similarity with the natural (faecal-oral) route of transmission of enteroviruses. Therefore, the present work aimed to investigate the effects of oral exposure to bovine enterovirus (BEV member of the *Picornaviridae* family) in an animal model through biochemical, immunological, histological and molecular analyses.

MATERIALS AND METHOD

Animals

The experimental procedures were conducted according to the Principles of Laboratory Animal Care (NIH publication nº. 80-23, revised 1996; http://www.nap.edu/readingroom/books/labrats/). Permission for the animal work was obtained from the Ethics Committee for Animal Experimentation of *Universidade Feevale* (protocol number 2.11.01.10.1643). All rats (male, 200-250g, 8 weeks old) were housed under conventional conditions with controlled temperature, humidity and light (22±3ºC, 12h light-dark cycle) and were provided with a standard commercial diet and sterile water. The animals were randomly divided in five groups: control (n=10), enterovirus (n=9), immunized (n=10), immunized+enterovirus (n=10) and streptozotocin (STZ) (n=6).

Exposure to bovine enterovirus

Animals from immunized and immunized+enterovirus groups were injected, via intramuscular with two doses of 0.3mL/kg of an emulsion containing $10^{5.75}CCID_{50}$/mL (50% cell culture infective dose) of bovine enterovirus (BEV -strain VG-5-27, grown on CRIB bovine cells in Dulbecco's Modified Minimum Essential Medium) and an oil adjuvant (mineral oil) (3:1; mineral oil, virus). The interval between the administrations prime and booster immunization was of 21 days. Seven days after the second immunization, animals from the enterovirus and immunized+enterovirus groups were challenged with 100mL of water experimentally contaminated with $10^{5.75}CCID_{5}0$/mL. Viral titers were determined by the Spearman method [14].

Streptozotocin model

Diabetes was induced in the STZ group by an intraperitoneal injection of 45mg/kg of streptozotocin (Sigma-Aldrich) dissolved in 0.1M citrate buffer (pH 4.5), as described previously [15]. Non diabetic animals were injected with citrate buffer alone. Right after the STZ administration, the animals received 5% glucose water for 24h in order to reduce death by hypoglycemic shock. In order to control the animal model, blood glucose levels were determined through a drop of blood from the tail vein, using a portable glucometer (Accu-Chec, Roche®) once a week for five weeks. After obtaining consecutive blood glucose levels higher than 300mg/dL, the animals were killed for blood and tissue collection.

Blood, tissue and stool collection

A pool of stools samples from each group (except for STZ group) was collected every day for four weeks after the oral exposure to enterovirus. Samples were stored frozen at -80ºC for viral analyses. Then, animals were anesthetized by an intramuscular injection of 75mg/kg of ketamin (Syntec®) and killed by decapitation. Samples of pancreas were immediately harvested and fixed in 4% neutral formalin solution for histological evaluation, while blood was collected in Becton Dickinson Vacutainer® tubes for glycemia, lipid profile and antibodies analyses.

Biochemical parameters

The glycemia determination was performed immediately after blood collection. The samples were then kept in -20ºC until the analysis of other biochemical parameters. These parameters included total cholesterol, c-HDL and triglycerides. Tests were performed using Cobas c111 (Roche®).

Detection of antibodies to glutamic acid decarboxylase (GAD)

GAD-specific autoantibodies were assayed by a quantitative sandwich enzyme immunoassay technique, using the commercial kit GAD1 ELISA (Cusabio Biotech, Wuhan, China), according to the manufacturer's instructions. Briefly, a 96-well plate was used, where 100µL of standard and sample were added in triplicate per well and incubated for two hours at 37ºC. After removing the liquid of each well, 100µL of biotin-antibody was added and incubated for one hour at 37ºC. Each well was aspirated and washed three times with wash buffer using an autowasher. Then, 100µL of HRP-avidin was added per well and incubated for one hour at 37ºC. The plate was washed three times again and 90µL of TMB substrate was added. The plate was incubated for 30 min. at 37ºC protected from light. After this, 50µL of stop solution was added to each well and the absorbance was read at 450nm within five min in a microplate spectrophotometer (Titertek). Data of autoantibodies were expressed as pg/mL.

Histological evaluation

Serial 7 µm thick sections of formalin-fixed, paraffin-embedded samples of fragments of pancreas were stained with haematoxylin and eosin (HE) and for granulated β-cell with the aldehyde fuchsin method [16].

For general evaluation, quantification of islet size and perimeter, images of pancreas were digitized using an Olympus CX41 microscope (40X) coupled to a CCD camera. The images obtained were measured using the Image-Pro Plus Software version 6.0.

Initially, islets were analyzed regarding to cellular infiltration and necrosis, as described previously [17]. A score of 0 corresponds to the absence of inflammation or necrosis; 1, incipient, focal inflammation or necrosis (only one or two foci in the whole section); 2, mild to moderate infiltration or necrosis (10-40% of section affected); 3, moderate infiltration (40-70% of section affected); 4, extensive areas of infiltration or necrosis (70-100% of the tissue section affected). Then, morphometric parameters as islet size and perimeter were estimated. Considering that in rodents, the core of the islets is formed by insulin-producing β cells (80% of the islet cells) surrounded by the three other cell types, namely α-, δ- and PP-cells [18], five central equal-sized areas of interest (AOIs) of 61440 μm^2 were delimitated in order to estimate de pancreatic β cell density. The total area was multiplied by the mean value of point-counted cells and divided by the value of the analyzed area (61440 μm^2) to estimate the β cell density. The cell nuclei located inside the square or intersected by the lower and/or right edge of the square were counted. The cell nuclei that were intersected by the upper and/or left edge of the square were not counted [19]. At least, five images of each islet were analyzed. The shape coefficient, also known as shape Z, was also used to evaluate differences in the shape of the pancreatic islets. This parameter is obtained using the following equation: $Shape\ Z = P/\sqrt{\hat{A}}$, where Shape Z is the shape coefficient, P is the perimeter, and Â is the area value [20].

Viral detection in stools

After the samples were thawed, the commercial kit RTP® DNA/RNA Virus Mini Kit (Invitek™, Germany) was used for the extraction of the viral RNA, according to the manufacturer's instructions. In order to obtain the amplification of BEV genome, a previous step of cDNA synthesis was carried out before amplification. It was performed using the High Capacity cDNA Reverse TranscriptionTM commercial kit (Applied BiosciencesTM, USA), with the aid of random primers and RNAse Inhibitor (Applied BiosciencesTM, USA), following manufacturer's instructions. The BEV sample used was the prototype strain VG-5-27, cultivated in CRIB cells [21] following routine protocols.

The real time PCR (qPCR) standardized for BEV was performed with the primer BEV POL3D, previously described [22], BEV POL3D-1 (5'-GCGTCGTACCCGTATGAGAT-3') and BEV POL3D-2 (5'-ATCACGCACGAACTTCCTCT-3'). qPCR reactions were conducted in a thermal cycler iQ5™ Bio-Rad (Biorad™, Hercules, California 94547, USA). For each 25 µL reaction, 12.5 µL of the mix were used, 1 µL of each primer (20 pM), 5.5 µL of DNAse/RNAse free sterile water and 5.0 µL of the nucleic acid extracted from each sample. Each reaction was composed of a denaturation cycle of 95°C by 10 min., followed by 40 cycles of 94°C for 20 s and 59°C for 1 min. The fluorescence data were collected during the annealing/extension step. After, a denaturing curve was made to check the specificity of amplification products (melting step between 55 and 95°C). No template control (NTC) and negative control were used in each run to confirm that there was no contamination in the assay. Melting curve analysis was done using High Resolution Melting electrophoresis (HRM) to verify PCR product specificity, since each viral species has a specific temperature (BEV; 82°C). For the quantification of viral particles by qPCR, BEV positive control standards with known titles of infective doses were used.

Statistical analysis

Statistical analysis was performed using one way analysis of

variance (ANOVA) followed by the Tukey post hoc test. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software. Data are expressed as mean ± standard deviation. Differences were considered statistically significant when the P value was < 0.05. For viral excretion, a descriptive analysis of data was performed.

RESULTS

Biochemical findings

A significant increase of blood glucose levels was observed in immunized, immunized+enterovirus and STZ groups when compared with control animals (P<0.01; P<0.01; P<0.001, respectively) (Fig. 1), indicating a potential role for enterovirus in increasing glycemia.

Concentrations of total cholesterol, c-HDL and triglycerides were also determined in the experimental groups. A statistical significant increase of total cholesterol was observed in immunized and immunized+enterovirus groups when compared with control animals (P<0.01), whereas no differences among groups were observed related to c-HDL and tryglicerides (Tab. 1).

Anti-GAD antibodies

The levels of anti-GAD antibodies in sera from experimental groups are shown in figure 2. A statistical significant increase of anti-GAD antibodies was observed in STZ group when compared to other groups (P<0.001).

Histological analysis

The histological evaluation using HE staining, revealed normal exocrine pancreas and arrange of the pancreatic islets, with no infiltrates or necrosis in all groups (Fig. 3A). Aldehyde fuchsin staining of pancreas showed normal islets with clusters of purple granulated β cells in all groups (Fig. 3B) except for STZ group, where marked degenerative changes of β cells in the islets were observed (Fig. 3C).

Regarding to morphometric measurements of islets size and β cells density, no statistical differences were found among groups, demonstrating that the viral exposure via oral route or immunization and STZ administration did not interfered in these parameters in this study (Tab. 2). However, statistical difference between the STZ group when compared to others was observed when analyzing the shape Z (Fig. 4), indicating that despite the normal islet size and pancreatic β cells density, the drug induced morphological alterations in the islets shape.

Viral excretion

Animals from enterovirus and immunization+enterovirus excreted the virus in stools continuously on the following days after ingestion of water experimentally contaminated with BEV (Fig. 5). Immunization+enterovirus group excreted higher

Table 1: Lipid profile of Wistar rats exposed to bovine enterovirus via oral route and immunization and streptozotocin (STZ)-induced diabetes.

Lipid profile (mg/dL)	Groups				
	Control	Enterovirus	Immunized	Immunized+ enterovirus	STZ
Total cholesterol	51.3± 7.9	56 ±10.7	61.6±9.9**	68.2±14**	49.8±8.3
c-HDL	42±10.3	32.5±4.6	37.7±6.2	37.3±6	33.1±9.6
Triglycerides	74.1±19.4	75.6 ±19.5	75.9±23.7	77±12	86.6±15

Data are expressed as mean±standard deviation. **P<0.01 vs. control group.

Table 2: Morphometric measures of pancreatic islets of Wistar rats exposed to bovine enterovirus via oral route and immunization and streptozotocin (STZ)-induced diabetes

Morphometric parameters	Groups				
	Control	Enterovirus	Immunized	Immunized+ enterovirus	STZ
Islet size (μm^2)	8.49E+05± 2.18E+05	9.01E+05 ±2.72E+05	1.1E+06± 2.33E+05	1.04E+06± 1.52E+04	8.36E+05± 2.04E+05
β cell density	151.2± 36.4	156.9±36.5	223.1±17.4	214.3±16.8	176.5±48.8

Data are expressed as mean±standard deviation.

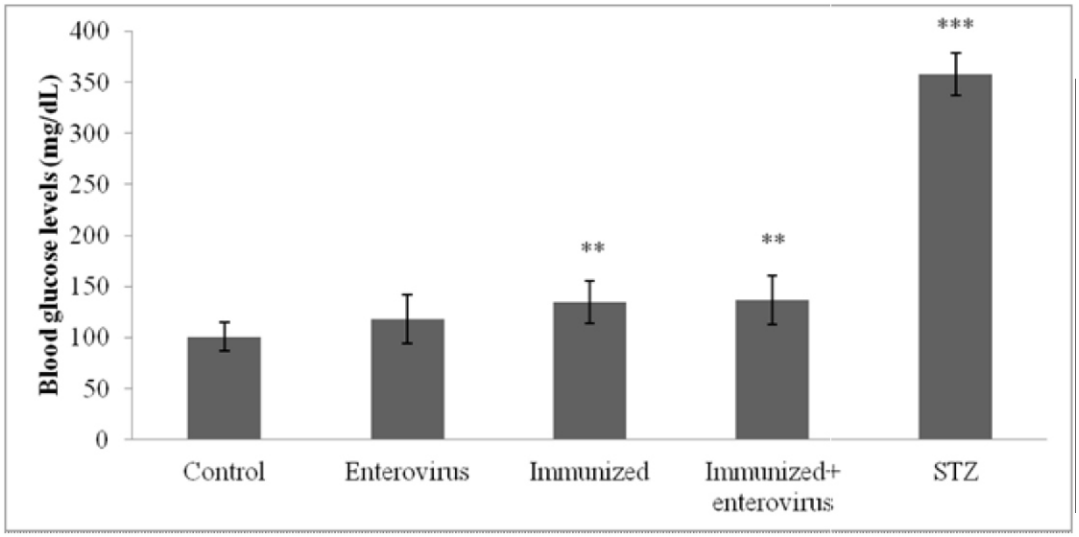

Figure 1: Blood glucose levels (mg/dL) in Wistar rats exposed to bovine enterovirus via oral route and immunization and streptozotocin (STZ)-induced diabetes. Data are expressed as mean±standard deviation. **P<0.01; ***P<0.001 vs. control group.

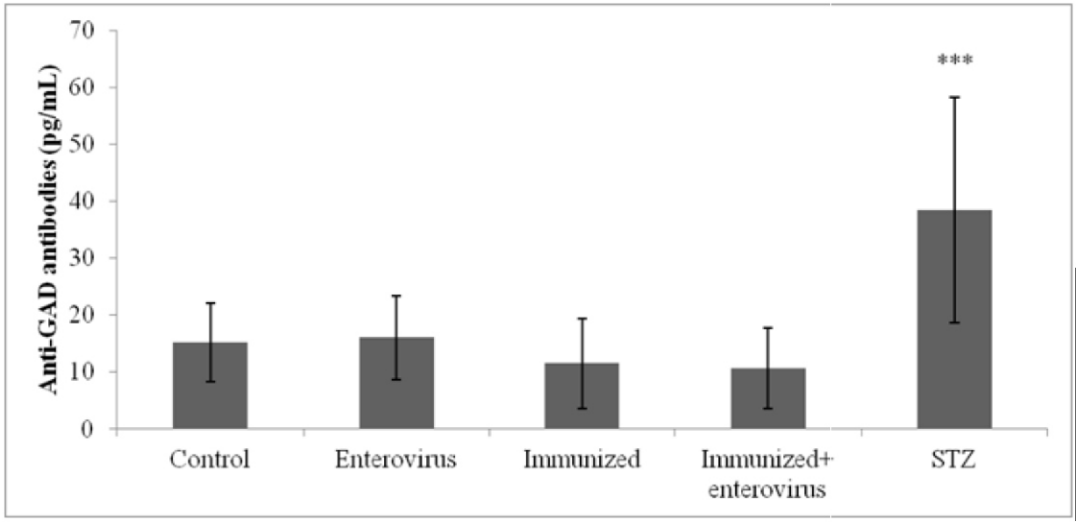

Figure 2: Anti-glutamic acid decarboxylase (GAD) antibodies of Wistar rats exposed to bovine enterovirus via oral route and immunization and streptozotocin (STZ)-induced diabetes. Data are expressed as mean±standard deviation. ***P<0.001 vs. all groups.

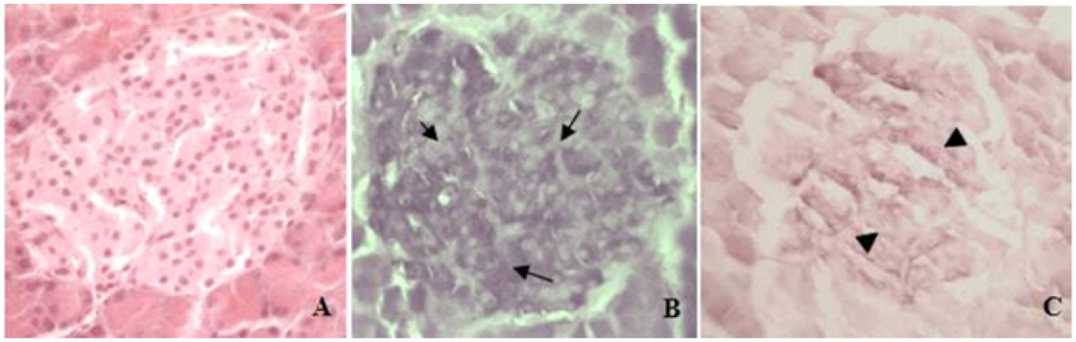

Figure 3: Histological sections of pancreas from Wistar rats. (A) Representative photomicrograph of pancreas stained with haematoxylin and eosin (HE), where no morphological alterations were observed. (B, C) Sections stained with aldehyde fuchsin. In B, representative photomicrograph of normal islet with granulated β cells (arrows) from control, enterovirus, immunized and immunized+enterovirus groups; in C, a β cell-degranulation was observed in the streptozotocin (STZ)-treated group (arrowheads). Magnification x400.

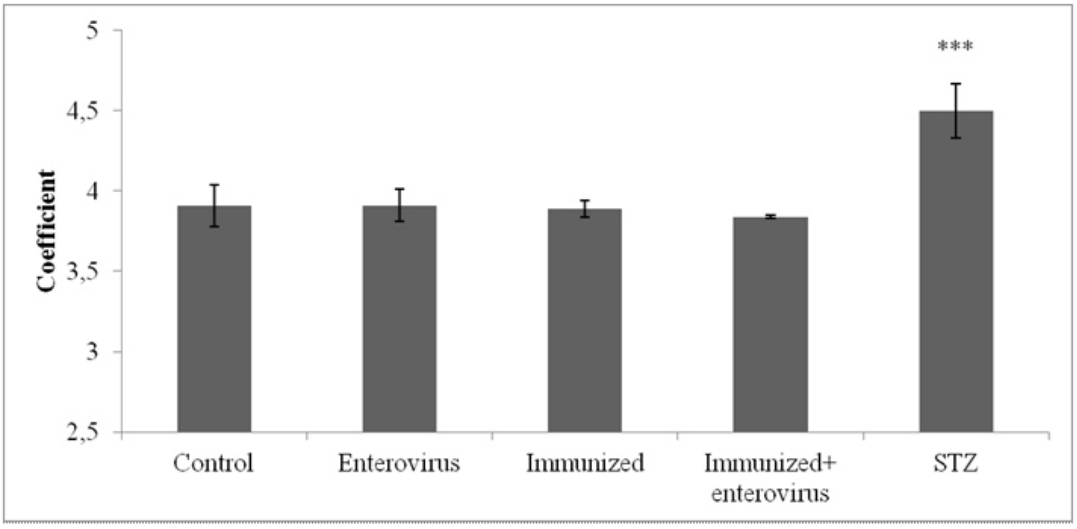

Figure 4: Shape Z of pancreatic islets of Wistar rats exposed to bovine enterovirus via oral route and immunization and streptozotocin (STZ)-induced diabetes. Data are expressed as mean±standard deviation. ***P<0.001 vs. all groups.

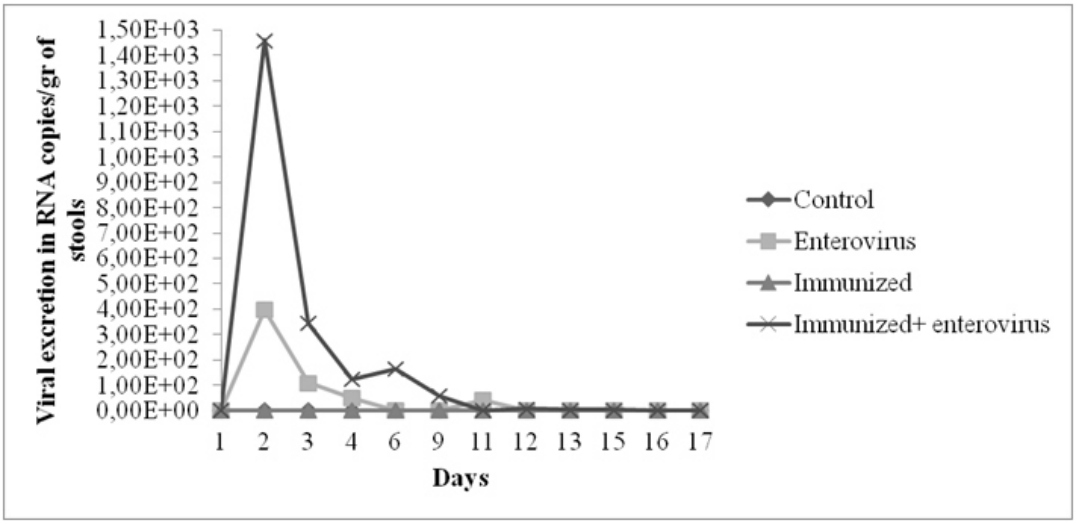

Figure 5: Viral excretion in stools Wistar rats exposed to bovine enterovirus via oral route and immunization. Continuous excretion of the virus in enterovirus and immunized+enterovirus groups until six days post exposure via oral route and lower quantities on the following days, whereas absence of the virus in control and immunized animals. Data are expressed as RNA copies/gr of stools.

quantities of copies of RNA/gr of BEV and for a longer period when compared to enterovirus group. Virus persistence was verified in the immunization+enterovirus group until 31 days post oral exposure to the virus (3.27E+01). No stool specimens from other groups were positive for BEV.

DISCUSSION

Although the findings from animal models may not necessarily translate to human disease [23], many studies in rodent models of spontaneous diabetes such as the NOD mouse, have significantly contributed to the understanding of the role of viruses in the pathogenesis of diabetes [24]. The NOD strain has many genes related to susceptibility to autoimmunity, which provides a fertile background onto which alternative major histocompatibility complex (MHC) genes confer alternative disease phenotypes [25]. However, since enteroviral infections have been strongly associated to islet autoimmunity, this work studied the effect of this environmental factor alone in an animal model with no genetic background for the disease.

In the present study, animals who received experimentally contaminated water with BEV did not present blood glucose impairment or pancreas morphology alterations, regarding to infiltrates, area and shape of islets and cell density. Other authors also found normal glucose levels and β cell function in Swiss mice 98 days after oral or intraperitoneal infection with different doses of coxsackie B virus [13].

Despite the normal islet morphology and low levels of anti-GAD when compared to control group, immunized animals presented significant higher levels of glycemia and total cholesterol, indicating a potential role for a non diabetogenic strain in T1D pathogenesis. It is known that immunization with an

adjuvant enhances the immune response [26], thus, the production of antibodies against the virus may have caused damages in a small number of insulin-producing cells in the immunized animals, without a market generation of specific antibodies against GAD. A number of β cells may have remained unrecognized and these cells were able to compensate for the deficiency to maintain glucose tolerance. Also, it has been proposed that enterovirus may provoke diabetes only when a preexisting mass of insulitis has accumulated [27], which may have compromised the development of overt T1D in this study.

Histological analysis evidenced alterations only in animals of the STZ group. Shape Z demonstrated that STZ administration induced morphological alteration in the shape of islets. Some studies have shown that STZ leads to a reduced number of islets and atrophy [28, 29]. Although area and cell density were similar to control group, aldehyde fuchsin staining showed destruction of pancreatic β cells in this group, confirmed by the anti-GAD antibodies assay, pointing to a loss of functionality of these cells, hence interfering in the blood glucose metabolism.

No alterations in blood glucose levels and in other parameters tested were observed in animals that ingested the experimentally contaminated water, indicating that one single exposure to BEV is not sufficient to trigger the autoimmunity which leads to diabetes in a short period of time. However, the involvement of viruses in the T1D pathogenesis cannot be disregarded, since latest evidence supports the hypothesis that enterovirus infections manifest in a slow rate of replication and persistence rather than acute infection [9]. Hence, prolonged infectivity with viruses, viral proteins or viral genome can lead to autoimmunity via constant activation of the immune response. After such polyclonal activation and proliferation of B cells, sometimes a monoespecific proliferation can emerge, accompanied by circulating immune complexes and eventually damage self-tissues [10].

Among others, there are two important factors to be considered in diabetes-induction in animal models by enterovirus exposure: timing of infection and diabetogenic viral strains. Timing of infection may interfere in the diabetic process since it determinates whether diabetes ensues [27, 30], along with the immune response to infection [31-33] and differences in the viral genome [23, 34]. Apparently, some kind of immunity is acquired with age, which usually moderates the severity of infection [13]. It has been demonstrated that some kind of intrinsic immunity of the gut matures with age, which provides a barrier to protect the host against overwhelming invasion by the pathogen [35]. Also, it has been reported that amino acid changes in the capsid and non-capsid proteins may be involved in the determination of diabetogenicity [36]. In encephalomyocarditis virus, a single point mutation in a recombinant virus genome induced diabetes in mice [37]. The mutation was located in the capsid protein VP1, causing an amino acid change and gain of viral diabetogenicity.

The viral detection in stools specimens revealed the excretion of BEV by the groups which ingested the contaminated water. Some studies in humans demonstrated the possibility of isolating viruses in stools even at 2 to 3 months after infection [38, 39]. In animals, the viral persistence in intestine was evidenced after 45 days post ingestion of virus-contaminated water [40]. Animals from the immunized group which ingested the water excreted higher quantities of the virus in stools than the enterovirus group, indicating that antibodies from the immunization may have enhanced the infection and hence, caused a greater elimination of the virus. A virus recognizes and binds one or several specific cell surface receptor(s), enabling the infection of the cell. Neutralizing antibodies prevent virus from infecting their target cells. In contrast, antibodies may increase the ability of viruses to infect their target cells, a phenomenon called "antibody-dependent enhancement of infection" [40, 41]. The occurrence of this phenomenon has been demonstrated with Dengue virus [42-44] and also with viruses from the *Picornaviridae* family [45-47]. Given the higher quantities of copies of viral RNA in the immunized animals, such phenomenon may have occurred in this group.

CONCLUSION

Most studies focus on the role of known viral diabetogenic strains in inducing T1D in susceptible mice. Although these studies have provided a great knowledge of the possible mechanisms which viruses may trigger diabetes, it is also relevant to carry out studies comprising the oral route of infection in animal models with no genetic background for the disease. In our study, one single exposure to bovine enterovirus was not sufficient to induce diabetes. However, the immunization may have enhanced the production of antibodies causing a minor destruction of insulin- producing β cells, hence leading to increased blood glucose levels. Such antibodies production may also have enhanced the infection by the virus, given the higher excretion of the virus in stools. Apparently, multiple factors including viral islet tropism, host susceptibility and additional environmental factors are required for diabetogenicity.

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