Effects of bovine enterovirus and type 1 diabetes on liver and kidney pyruvate kinase activity in an animal model

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ABSTRACT. Type 1 diabetes (T1D) is an autoimmune disease characterized by the selective destruction of pancreatic beta cells. In addition to genetic factors, enteroviruses have been considered the main environmental factor involved in this pathology. Therefore, the objective of this study was to evaluate the effects of streptozotocin-induced diabetes and bovine enterovirus (BEV) on liver and kidney pyruvate kinase activity in rats. Fourteen male Wistar rats were divided in three groups: control, diabetes and a third group, which was fed with water experimentally contaminated by BEV. Increased blood glucose levels were found in both diabetes and enterovirus groups, whereas there were no alterations in the lipid profile. A reduced pyruvate kinase activity was observed in the liver and kidney of animals from diabetes and enterovirus groups. Under our experimental conditions, the ingestion of water experimentally contaminated by BEV induced alterations in glycaemia, and also interfered in the pyruvate kinase activity in liver and kidney of the rats, which might be one of the possible mechanisms involved in the T1D development.

Keywords: animal model; autoimmune disease; enzymatic activity.

Introduction

Type 1 diabetes (T1D) is a chronic disease which results from the impaired metabolism of glucose, leading to a high concentration of glucose in the circulation (Antonelli, Ferrari, Di Domenicantonio, Ferrannini, & Fallahi, 2015). It is characterized by the progressive and selective destruction of pancreatic beta cells in genetically predisposed individuals during childhood and adolescence (Richardson, Willcox, Bone, Morgan, & Foulis, 2011), although some epidemiological studies have indicated that its incidence may be comparable to adults in some countries (Molbak, Christau, Marner, Borch-Johnsen, & Nerup, 1994). As for other autoimmune diseases, diabetes is triggered by the interaction between genetic and environmental factors (Bason et al., 2013). Among the environmental factors that might be involved in the development of the diabetic process are diet, sedentary, exposure to toxins and viral infection. The last one is most commonly caused by enterovirus (Roivainen & Klingel, 2009).

The enteroviral infections, caused by an RNA virus from the Picornaviridae family, are responsible for most viral diseases which affect humans, considering that one billion people are infected annually worldwide (Oberse & Pallansch, 2003). Enteroviruses are transmitted via the fecal-oral route and replicate primarily in the gut. The systemic infection may lead to the dissemination to other target organs (Antonelli et al., 2015; Hankaniemi et al., 2017). The viruses from the Enterovirus genus do not have a lipid envelope and their genome is encoded as single-stranded RNA, which is protected by an icosahedral capsid (Nasri et al., 2007). These viruses are tolerant to residual chlorine from sewage treatment and to a wide range of temperatures and salinities, which facilitates their survival in water resources (Gregory, Litaker, & Noble, 2006).

Several studies in humans have demonstrated the association between enteroviral infection and T1D (Richardson et al., 2011; Salvatoni et al., 2013; Badia-Boungou et al., 2017). Such association has also been shown in animal models (Yoon, Austin, Onodera, & Notkins, 1979; Horwitz, Ilic, Fine, Balasa, & Sarvetnick, 2006).
The enteroviruses might initiate or accelerate the pathological events that lead to T1D through several mechanisms. First, the pancreatic beta cells might be destroyed directly through a cytolysis induction by the virus. Second, a less aggressive infection could cause an inflammatory reaction in the islets, leading to subclinical levels of beta cell destruction and subsequent antigen release, which activates autoreactive T lymphocytes. Alternatively, cross-reactive T cells could be induced, which occurs when viral and host antigens share the same antigenic determinants (Massilamany, Koenig, Reddy, Huber, & Buskiewicz, 2016).

Pyruvate kinase catalyzes the final step in the glycolytic pathway. The reaction product of the biochemical reaction is the pyruvate, which is involved in a variety of metabolic reactions. Thus, the pyruvate kinase may be considered a key enzyme not only for the glycolytic pathway but also for the entire cellular metabolism (Feksa, Cornelio, Vargas, Wyse, Dutra-Filho, Wajner, & Wannmacher, 2003; Wang, Zhao, & Liu, 2017). The inhibition of pyruvate kinase implies in the decreased synthesis of pyruvate, which can consequently induce cell death (Valentini, Chiarelli, Fortin, Speran, Galizzi, & Mattevi, 2000). There are at least four known isozymes of pyruvate kinase in mammals, which present different properties and tissue distribution: the isozyme L (liver), the isozyme M1 (brain, muscle), the isozyme M2 (kidney) and the isozyme R (erythrocyte) (Hall & Cottam, 1978). Since the liver is the primary organ for glucose metabolism (Chandrasekaran, Swaminatthan, & Chatterjee, 2010), it is important to evaluate the activity of this enzyme in this organ. Therefore, this study aimed at evaluating the effects of bovine enterovirus infection and diabetes on pyruvate kinase activity and some biochemical parameters in animal model.

**Material and methods**

**Animals**

A total of 14 male Wistar rats aged 60 days from *Universidade Feevale*, Novo Hamburgo, Brazil, were used in the experiment. The animals were kept at 22 ± 3ºC under a 12 hours light-dark cycle. The animals had access to water and to a standard commercial feed *ad libitum*. All experiments followed the 'Principles of Laboratory Animal Care' (NIH publication nº. 80-23, revised 1996; http://www.nap.edu/readingroom/ books/labrats/). The research protocol was approved by the Ethics Committee for Animal Experimentation of *Universidade Feevale*. The animals were randomly divided in three groups: control (n = 5), diabetes (n = 4) and enterovirus (n = 5).

**Exposure to bovine enterovirus**

The animals from the enterovirus group were fed during a week with water experimentally contaminated with 100 infective doses of bovine enterovirus (BEV, strain VG-5-27, grown on CRIB bovine cells) in DMEM (Dulbecco’s Modified Minimum Essential Medium), diluted in 500 mL of autoclaved water. The animals from the control and diabetes groups received a solution containing 0.1M citrate buffer, pH 4.5, in water. Viral titers were determined by the Spearman method (Spearman, 1908).

**Streptozotocin-experimental diabetes model**

Diabetes was induced by an intraperitoneal injection of 55 mg kg⁻¹ of streptozotocin (STZ) (Sigma-Aldrich) dissolved in 0.1M citrate buffer (pH 4.5), as described previously (Rodrigues, Figueroa, Mostarda, Heeren, Irigoyen, & Angelis, 2007). Non diabetic animals were injected with citrate buffer alone. Right after the STZ administration, the animals received 5% glucose water for 24 hours in order to reduce death by hypoglycemic shock. Thus, to control the animal model, blood glucose levels were determined through a drop of blood from the tail vein, using a portable glucometer (Accu-Chek, Roche®).

**Blood and tissue samples**

At 45 days of the experiment, the animals were killed by decapitation. Liver and kidneys were immediately removed, whereas blood was collected in Becton Dickinson Vacutainer® tubes for glycaemia and lipid profile analyses.

**Biochemical parameters**

The glycaemia determination was performed immediately after blood collection. Subsequently, the samples were then kept in -20°C until further analyses of other biochemical parameters, which included total cholesterol, c-HDL and triglycerides. Tests were performed using Cobas c111 (Roche®). Previous
studies have demonstrated that values for glycaemia in control Wistar rats vary from 93 to 104 mg dL⁻¹ (Bhansali et al., 2015; Dalzochio et al., 2015). The procedure performed to determine the pyruvate kinase activity was previously described by Rieger, Rech, Feksa, and Wannmacher (2008). After the removal, liver and kidneys were weighted and individually homogenized in a buffer solution containing 0.32 mol L⁻¹ sucrose, 1 mmol L⁻¹ EGTA and 10 mmol L⁻¹ Tris-HCl, pH 7.4 (1:10, w:v⁻¹). The homogenate was centrifuged for 10 minutes at 800 g at 4°C in a Sorval refrigerated centrifuge (Thermo Fisher Scientific, Pittsburg, KS, USA). The pellet was discarded, and the supernatant was centrifuged for 15 minutes at 10 000 g. The supernatant from this last step was collected for determination of pyruvate kinase. Pyruvate kinase activity was determined according to the method described by Leong, Lai, Lim and Clark (1981), with modifications. The incubation medium was composed by 20 mmol L⁻¹ MgCl₂, 150 mmol L⁻¹ KCl, 5.0 mmol L⁻¹ ADP, 0.1 mol L⁻¹ Tris-HCl buffer, pH 7.5, 0.16 mmol L⁻¹ NADH, 7 units LDH, 0.2% (v:v⁻¹) Triton X-100 and 10 μL mitochondria free supernatant in a final volume of 0.5 mL. After 30 minutes of preincubation, the reaction started by the addition of 1.0 mmol L⁻¹ phospho(enol) pyruvate. The NADH consume was measured every 30 seconds during two minutes at 340 nm. The protein content was determined according to Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin as standard. Results of liver and kidney pyruvate kinase activity are expressed as nmol pyruvate formed min⁻¹ mg protein⁻¹.

**Statistical analysis**

Statistical analysis was performed by a 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 ± SD. Differences were considered statistically significant when p < 0.05.

**Results**

An increase in blood glucose levels was observed in diabetes and enterovirus groups when compared to the control group: F (2, 10) = 690.04; p< 0.001 and p< 0.05; respectively, indicating the success of the STZ application and a potential role for enterovirus in increasing glycaemia (Figure 1). Regarding the lipid profile, no differences among groups were observed, demonstrating that in this experiment, STZ and enterovirus did not induce alterations in these parameters (Table 1).

![Figure 1](image.jpg)

*Figure 1. Effects of streptozotocin-induced diabetes and bovine enterovirus on blood glucose levels (mg dL⁻¹) in Wistar rats. Data are expressed as mean ± SD. The statistical analysis performed was one-way ANOVA and Tukey test. *p < 0.05; **p < 0.001 compared to the control.*

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetes</th>
<th>Enterovirus</th>
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<tbody>
<tr>
<td>Cholesterol</td>
<td>54.8 ± 11.8</td>
<td>42.3 ± 6.8</td>
<td>48.6 ± 5.5</td>
</tr>
<tr>
<td>c-HDL</td>
<td>59.6 ± 7.9</td>
<td>26.7 ± 4.7</td>
<td>54.4 ± 4.1</td>
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<tr>
<td>Triglycerides</td>
<td>111.8 ± 11.5</td>
<td>123.2 ± 31.4</td>
<td>102.6 ± 24.4</td>
</tr>
</tbody>
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*Data expressed as mean ± SD. The statistical analysis performed was one-way ANOVA. No statistical differences among groups were observed.*
As shown in Figure 2, a statistically significant reduction of pyruvate kinase activity in the kidney was observed in diabetes and enterovirus groups when compared to the control: F(2, 11) = 51.14, p < 0.001 for both groups. When analyzing the pyruvate kinase activity in the liver, a statistically significant reduction was also observed in diabetes and enterovirus groups when compared to the control: F(2, 11) = 5.24, p < 0.05 for both groups (Figure 3).

**Figure 2.** Effect of streptozotocin-induced diabetes and bovine enterovirus on kidney pyruvate kinase activity in Wistar rats. Results are expressed as nmol of pyruvate kinase per min per mg of protein. Data are expressed as mean ± SD. The statistical analysis performed was one-way ANOVA and Tukey test. **p < 0.001 compared to the control.

**Figure 3.** Effect of streptozotocin-induced diabetes and bovine enterovirus on liver pyruvate kinase activity in Wistar rats. Results are expressed as nmol of pyruvate kinase per min per mg of protein. Data are expressed as mean ± SD. The statistical analysis performed was one-way ANOVA and Tukey test: * p < 0.05 compared to the control.

**Discussion**

Several lines of studies in humans and in animals suggest the role of enterovirus in the development of T1D. In this study, an animal model was used to investigate the possible role of bovine enterovirus in the pyruvate kinase activity in the liver and kidney and consequently, in diabetes. The animals who received STZ and the enterovirus inoculated by the oral route presented an increase in glycaemia that was statistically significant when compared to the control group. Although the diabetes group had presented higher levels of blood glucose than the enterovirus group, a role for this virus in the diabetic process should be considered. Previously, Dalzochio et al. (2015) have also demonstrated the potential of immunization with enterovirus in increasing blood glucose levels in Wistar rats. Nonetheless, the authors have also found no blood glucose impairment and morphological alterations in the islets of animals infected with enterovirus via the oral route. One important aspect to be considered is that after the development of the autoimmune process which leads to diabetes, clinical manifestations of the disease appear when about 80% of beta cells have been destroyed (Liu & Eisenbarth, 2002). Given the slow progression of the disease, it seems reasonable that, in this study, the enterovirus group did not present blood glucose levels as high as the streptozotocin-induced diabetes animals. In addition, some studies which have shown the relation...
between diabetes and enterovirus were performed using genetically predisposed animals – nonobese diabetic (NOD) mice (Yoon et al., 1979; Horwitz et al., 2004). These studies also used the intraperitoneal route of infection mainly because this infection route causes consistently more morbidity and proportional mortality than the oral infection route (Bopegamage et al., 2005). Nevertheless, the intraperitoneal injection is an inoculation route that does not comprise the way the infection occurs in humans. Consequently, studies on the effects of viral infection occurring through the inoculation by contaminated water are necessary.

Regarding the lipid profile, in the past years, some studies investigated a role of diabetes in the lipoprotein's concentrations, since dyslipidemia represents a risk factor for health. The relation between diabetes and the increase of lipoproteins has been demonstrated (Jenkins, Steele, Janus, Santamaria, & Best, 1992); however, this finding is controversial in other studies (Rainwater, Maccluer, Stern, Vanderbe, & Haffner, 1994; Chico, Pérez, Caixas, Ordoñez, Pou, & De Leiva, 1996). In this study, using an animal model and streptozotocin-induced diabetes, no alterations were found. Accordingly, Dalzochio et al. (2015) have also observed no effects in the lipid profile of animals exposed to the enterovirus and streptozotocin-induced diabetes.

There are conflicting data regarding pyruvate activity in diabetes. The pyruvate activity and RNAm expression is reduced in adipose tissue (Belfiore, Rabauzzo, Napoli, Borzi, & Lo Vecchio, 1975), in cultures of pancreatic beta cells in subjects with T1D (Lupi et al., 2004), as well as in animal models of diabetes with insulin impairment (Kondoh et al., 1992). Conversely, a slightly increase (Diamant & Shafrir, 1978) and no change (Rossi, Sánchez-Arias, & Feliu, 1990) in the pyruvate kinase activity have also been reported. These observations are important since it has been described that insulin activates pyruvate kinase (Parks & Drake, 1982). In the present study, the enterovirus ingestion and the administration of STZ caused a significantly reduction in liver and kidney pyruvate kinase activity, indicating that the ingestion of water contaminated with bovine enterovirus might inhibit the pyruvate kinase activity in kidney and liver of rats, in the same way as in streptozotocin-induced diabetes, which interferes in the glycolysis pathway. In studies performed with liver pyruvate activity, a decreased expression of pyruvate was observed in diabetic rats due to low levels of insulin (Sereday et al., 2004; Celik & Ergodan, 2008). Data from these studies corroborates with our observations, since a reduced pyruvate kinase activity was detected in animals of the diabetes and enterovirus groups. Thus, both T1D and enterovirus exposure influence in the rats’ pyruvate activity.

**Conclusion**

Our results suggest that enteroviruses might be related to disorders in the glucose metabolism. It was possible to evidence the potential of enterovirus in interfering in the liver and kidney pyruvate activity. Because enteroviruses are widely distributed in the environment and are also a common cause of infections in people, their role in T1D should be considered. Nevertheless, it is necessary to perform studies focused on enterovirus orally inoculated, as well as the use of techniques to verify the enterovirus loads needed to cause infection, in order to elucidate the possible mechanisms in which they act in the diabetic process.

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