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A Newly Developed Saliva-Based Elisa to Determine Immune Response to Milk Proteins in Young Diabetic Jordanians: Lack of Association for Infantile Feeding Practices

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ABSTRACT

There is growing worldwide interest in the identification of potential environmental factors that may trigger the pancreatic autoimmune process in genetically susceptible individuals. The possible immunogenicity of Bovine Serum Albumin (BSA) and modified and heat processed BSA with bovine insulin (mBI-BSA) was examined. Fifty diagnosed diabetic children under the age of 14 years and their unrelated age and gender matched control subjects were identified. Serum and saliva samples were collected. IgG and secretory IgA antibodies to BSA as well as to native and heat processed mBI-BSA were determined. Data on participants' breastfeeding and early infant feeding practices were collected using a constructed questionnaire developed previously. Heated mBI-BSA (70ºC/5min) has shown the highest relative change in both IgG and sIgA titre levels, whereas mBI-BSA has also shown a substantial relative change in antibody titre levels compared to that of untreated BSA. Mean log effect of mBI-BSA on the titre levels of both IgG and sIgA antibodies in both DC and NDC has increased by approximately 1.3 fold of that of unheated or modified BSA (P = 0.000 and 0.114, respectively). The mean log effect of heat processed mBI-BSA at (70ºC/5min) on IgG and sIgA titre levels in both groups has also increased by nearly 1.5 of that of native BSA (P = 0.000) and 1.1 fold of that of native mBI-BSA (P = 0.000). There were no immune responses to mBI-BSA heated at 70 ºC/10min, 80ºC/5min and 60ºC/5min). There were significant correlations between DC (60%) and NDC (25%) for the positivity of CRP (P = 0.05).

The saliva-based ELISA system may be a useful proxy of immune responses and may constitute new grounds for the ongoing dietary intervention trials. The modification and thermal processing procedure of BSA is a merely sophisticated system in eliciting immune responses and is neither limited to diabetic patients nor associated with breastfeeding or early infant feeding practices, and may reflect unspecific defect of the immune system.

Keywords: Autoimmune, Immune response, Feeding practice, Gut maturity, Protein, Modification, Heat, Diabetogenicity.

INTRODUCTION

Although a number of different complex mechanisms of pancreatic beta cell destruction are operative in type 1 autoimmune diabetes (Kawasaki et al. 2004), none of the potential primary genetic or environmental factors determinants of type 1 autoimmune diabetes has been established unequivocally (Akerblom et al., 2005; Nakayama et al., 2005). Nonetheless, bovine insulin is implicated as a putative primary autoantigen in animals (Atkinson et al., 1986; Kimpimaki et al., 2002), bovine

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insulin is a small protein (Harfenist and Craig, 1952; Sanger, 1958) and is unable to elicit immune response by itself. Rather it requires a linkage to a large immunogenic carrier, such as Bovine Serum Albumin (BSA) (Harlow and Lane, 1988).

The present study focuses on determining immune responses to modified and heat processed milk proteins particularly BSA and bovine insulin by using a newly developed saliva-based enzyme linked immunosorbent system (ELISA) in young Jordanian diabetic children. This will be with regard to their breastfeeding and early feeding practices of participants.

RESEARCH DESIGN AND METHODOLOGY

The Population Sample

The cohort of this case-control study consisted of 50 (36 females and 14 males) Jordanian diabetic children (DC) aged 14 years or less. Participants were identified by The National Centre for Diabetes, Endocrinology and Genetics, Jordan, and 50 (28 females and 22 males) unrelated, age and sex matched non-diabetic children (NDC) were also identified. Diagnosis of diabetes was confirmed by the medical history of the diabetic children, including the presence of clinical symptoms and the need for insulin therapy right after diagnosis. Participants were unrelated to those with diabetes or to each other. Control subjects were age and gender matched. Data on breastfeeding and early infant feeding practices were collected using a constructed questionnaire.

Ethical approval was granted by the Human Research Ethics Committee of the University of Western Sydney as well as by the authorities of all Jordanian participating institutions. All subjects, or their parents when appropriate, gave either a written informed consent or a witnessed verbal consent when appropriate.

Blood and Saliva Samples Collection

A licensed phlebotomist drew 10ml venous blood samples during routine consultation visits to the Hospital Outpatients Clinics by venipuncture into a clot (red top) test tube. Whole blood samples were stored overnight at 4ºC and then centrifuged at 4ºC/1000 rpm/15 min. Serum aliquots were stored frozen at -20ºC (Jonstone and Thrope, 1996). Two hemolyzed blood serum samples were discarded.

Twenty saliva samples were collected from age-matched DC and their age and sex matched controls. Participants with periodontal diseases or who had undertaken major dental work and/or those prone to respiratory infections were excluded. To minimize the potential of saliva contamination particularly from dairy products, whole unstimulated samples were collected at least 30 minutes after the last meal or drink. Participants were asked to rinse their mouth thoroughly with water 10 minutes prior to sample collection.

Participants were encouraged to relax and visualize their favourite foods. Participants were seated comfortably (Miletic et al., 1996) and saliva samples (5-10 ml) were drawn by either passive slavering into a sterile swap tube or by a sterile plastic transfer suction pipette. Saliva samples were clarified by centrifugation at 4ºC/1000 rpm/15min (Castro et al., 2004). Aliquots were stored frozen at -20ºC. To break down mucopolysaccharides saliva, samples were exposed to a single freeze-thaw cycle (Worthman et al., 1990; Shirtcliff et al., 2000).

Preparation of the Testing Antigens

Bovine Serum Albumin (BSA) and Bovine Insulin (BI) (Sigma-Aldrich, MO, USA) were reconstituted in double deionised ultrafiltered water (0.18µm) and were filtered through 0.22µm filter (Millipore, UK). Bovine serum albumin (1mg/ml) was modified with an equal

volume of BI (1mg/ml) under strictly aseptic physiological conditions (37ºC/24h/25rpm, pH 7.4).

Modified BSA with BI (m-BI-BSA) was subjected to water bath heat processing under the same conditions at 40ºC/10 min, 50ºC/5 min, 60ºC/5min, 60ºC/15min, 70ºC/5 min, 70ºC/10min and 80ºC/5min (Al-Domi et al., 2004). Since this study was carried out in Jordan and to verify our developed spectrophotometric assay, the modification and heat processing of mBI-BSA were conducted strictly under the same condition (Al-Domi et al., 2004).

Enzyme Linked Immunosorbent Assay (ELISA)

A newly developed ELISA was used to determine serum IgG and sIgA tire levels. In brief, 96-well, high-binding flat-bottom microtitre plates (Greiner-Bio-One GmbH, Germany) were coated with 50 µl of (1 µg/ml) native BSA, native mBI-BSA, and mBI-BSA heat processed at different temperatures and various exposure times (40ºC/10 min, 50ºC/5 min, 60ºC/5min, 60ºC/15min, 70ºC/5 min, 70ºC/10min and 80ºC/5min) in (0.1M phosphate-buffered saline (PBS), pH 7.4, buffer 1) (Sigma-Aldrich, St Louis, USA). Plates were incubated for 12 hours at 4 ºC.

The coated plates were washed with 0.05% Tween 20 in buffer 1 (buffer 2) five times. The plates were blocked with 100µl (1%) normal sheep serum (DakoCytomation, Denmark) thermally treated at 50ºC/5min in buffer 2 and were incubated for one hour at 37 ºC), then were washed with buffer 2 three times. Serum and saliva specimens were thawed and plates were coated with 50µl of both serum and saliva specimens. Two-fold dilution in buffer 1 was carried out for each of the specimens under the same conditions on the same plate this to avoid interassay variability. Coated plates were sealed and incubated for one hour at 37ºC followed by washing with buffer 2 three times.

Plates were coated with 50µl polyclonal alkaline-phosphatase-conjugated affinity purified rabbit anti-human IgG specific for Gamma-chains and alkaline-phosphatase-conjugated affinity purified rabbit anti-human IgA specific for Alpha-chains antisera (DakoCytomation, Denmark) diluted 1/3000 in buffer 2, incubated for one hour at 37ºC and washed with buffer 2 four times. Liquid p-nitrophenylphosphate substrate (50µl) (Sigma-Aldrich, St Lois, MO) was added and the plates were incubated for six hours at 37ºC. Reaction was stopped by adding 1 M NaOH and the end-point was measured at 405nm (ELX 800, Bio-Tek Instruments, Inc.; Kcjunior software). Duplicates varying by more than 5% error were retested. All plates were sealed prior to incubation, and incubation was undertaken in a humidified incubator with a preset temperature.

Optical densities were subjected to point-to-point analysis. The cut-off point was determined as blank Optical Density [OD] *2. Serum and saliva titres were expressed as the 50% of the reciprocal dilution factor of OD just above the cut-off point. Titres less than or equal to the cut-off point were assumed zero.

Statistical Analysis

All data were analysed using the Statistical Package for Social Sciences (SPSS). Assuming normally distributed errors, the data fit a linear regression model and the bivariate correlations procedure was used to compute Pearson's correlation coefficient and Paired-Samples T-test, independent-samples T-test and multivariate analysis of variance (ANOVA) parametric statistics were computed for serum and saliva logarithmic (log) titre levels.

Based on the values of a set of predictor variables, the logistic regression model was used to estimate Odds Ratios (OR) with 95% Confidence Interval (CI) for each

of the independent variables in the model. Data were considered statistically significant at $P<0.05$.

RESULTS

Figure 1 delineates relative change in the serum IgG and secretory IgA antibody titre levels produced to native Bovine Serum Albumin (BSA), and native and heat processed modified BSA with bovine insulin (mBI-BSA) in both DC and NDC, with reference to that of BSA. While mBI-BSA has shown a substantial relative change in antibody titre levels, different heat treatment temperatures and exposure times have exhibited a wide range of changes in IgG and sIgA immune responses. Heat processed mBI-BSA (70ºC/5min) has shown the highest relative change in both IgG and sIgA antibody titre levels. Heat processing of mBI-BSA at 40ºC/10min, 60ºC/15min, 70ºC/10min and 80ºC/5min has shown a negative relative change in the IgG and sIgA antibody titre levels, whereas heat processing of mBI-BSA at 50ºC, 60ºC and 70ºC for five minutes has shown a positive trend.

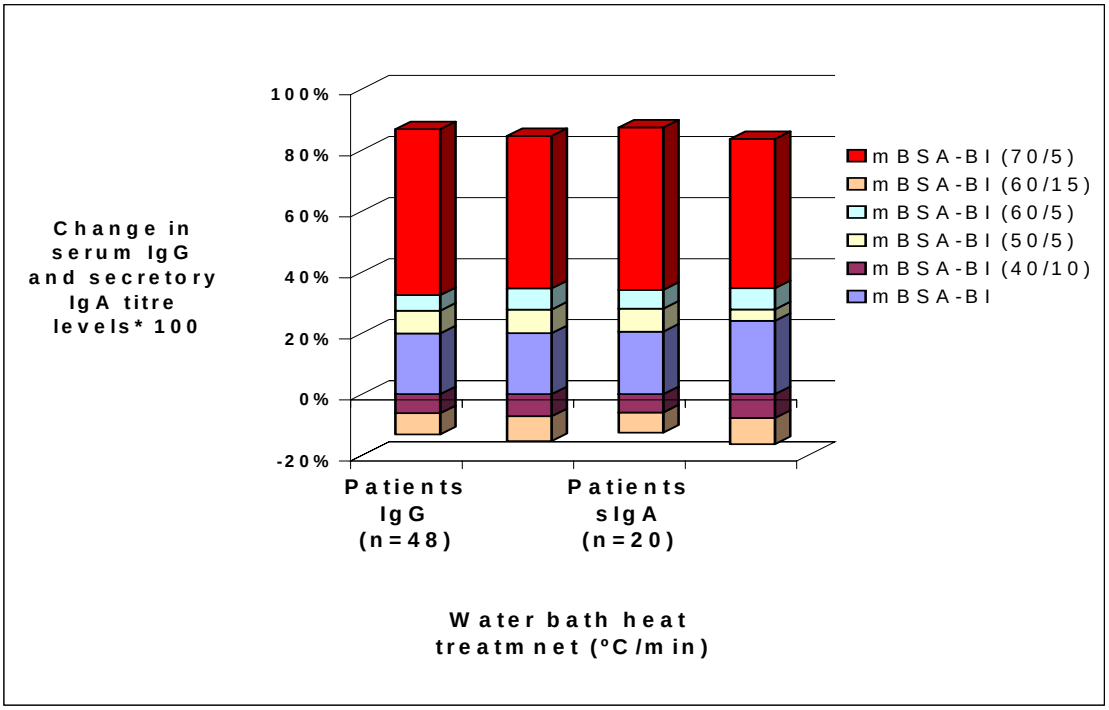
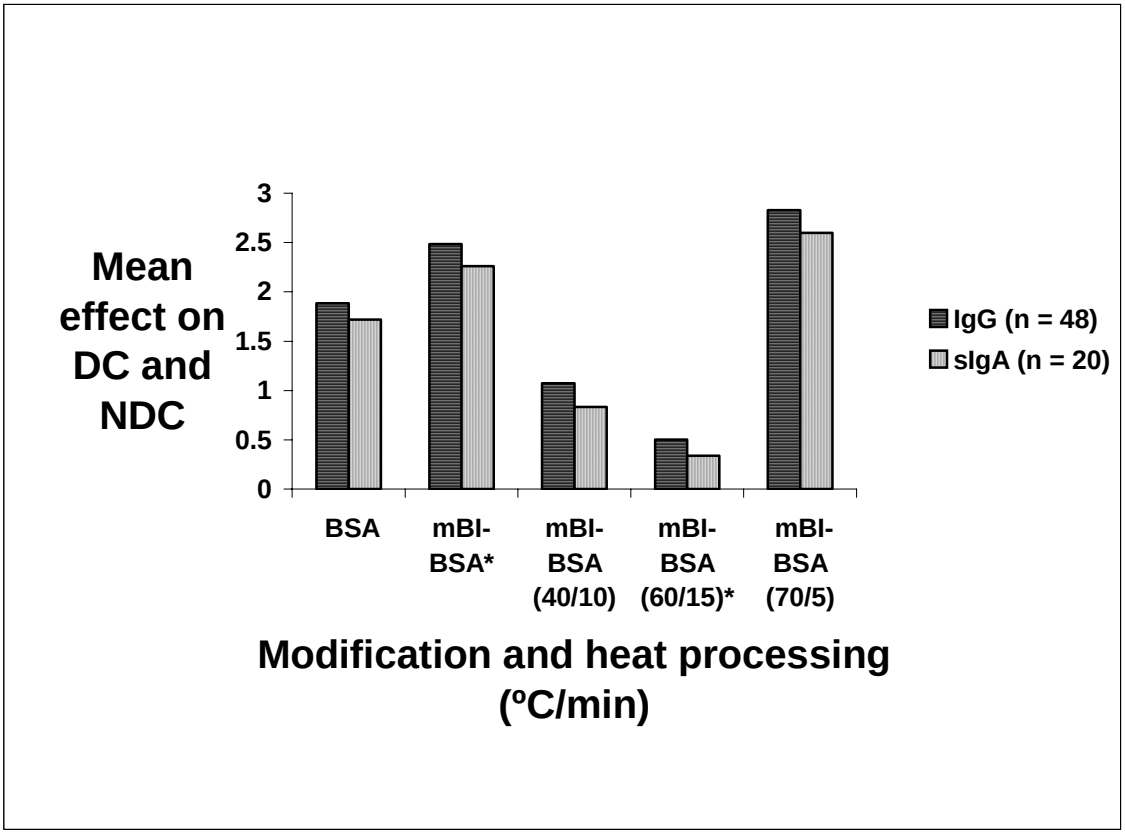


Figure (1): Percent change (*100) in serum IgG and secretory IgA (sIgA) titre levels of diabetic children produced to modified and heat processed Bovine Serum Albumin (BSA) with bovine insulin (mBI-BSA) at different temperatures and exposure durations (70ºC/5min, 60ºC/15min, 60ºC/5min, 50ºC/5min, 40ºC/10min and untreated mBSA-BI). Relative change calculated in reference to that produced against untreated BSA. Percent change calculated in reference to IgG and sIgA antibody titre levels produced against untreated BSA.



* The effect of mBI-BSA (60ºC/15 was statistically insignificant on sIgA antibody titre levels at P<0.05.

Figure (2): Comparison between the sample paired test mean effect that native Bovine Serum Albumin (BSA) and modified and heat processed BSA with bovine insulin (mBI-BSA) have on both logarithmic (log) serum IgG and secretory IgA (sIgA) antibody titre levels in both diabetic and non-diabetic Jordanian children aged 14 years or less. Mean effect was statistically significant at P<0.05 for all heat processed and modified BI-BSA.

Figure 2 compares logarithmic (log) mean effect that native Bovine Serum Albumin (BSA) and native and heat processed mBI-BSA have on both serum IgG and secretory IgA antibody titre levels in diabetic and non-diabetic Jordanian children aged 14 years or less. The mean log effect of mBI-BSA on the antibody titre levels of both IgG and sIgA antibodies in DC and NDC has increased by approximately 1.3-fold of that of unheated or modified BSA (P = 0.000 and 0.114, respectively). The mean log effect heat processed mBI-BSA at 70ºC/5min has on IgG and sIgA antibody titre levels in both DC and NDC has also increased by approximately 1.5-fold of that of native BSA (P = 0.000) as well as 1.1-fold of that of native mBI-BSA (P = 0.000).

In addition, there was a significant difference for the increase in mean log serum IgG antibody titre levels produced to mBI-BSA heat processed at 50 and 60ºC/5min (P = 0.000) as well as to sIgA antibody titre

levels produced against mBSA-BI heat processed at 50ºC/5min (P = 0.001). This was not significant to mBI-BSAS heat processed at 60ºC/5min (P = 0.155). Interestingly, there were neither humoral nor secretory immune responses to mBI-BSA heated at 70 ºC/10min and 80ºC/5min. Furthermore, there were significant correlations between DC (60%) and NDC (25%) for the positivity of CRP (P = 0.05, Phi = 0.21), given the normal levels of ≥ 6 mg/l (Hayashi et al., 1970; Roitt et al., 2001).

Titre levels of IgG antibodies produced to native and heated mBI-BSA at 70ºC/5min were strongly associated with the diabetic and control group (r = -0.78 and -0.65, P = 0.000, respectively); IgG antibody titre levels to heat processed mBI-BSA at 50 and 60ºC/5min were also positively associated (r = -0.45, -0.43 and -0.32, P = 0.00, respectively). Similarly, sIgA antibody titre levels produced to heated mBI-BSA at 70ºC/5min were strongly associated with the diabetic and control group (r = -0.77, P = 0.000) that was not significant for native mBI-BSA.

Table 1 shows that there were statistically significant differences for tests of between-subjects effect in DC and NDC for interactions between IgG and sIgA antibody titre levels produced to unheated and not modified BSA, and native and heat processed mBI-BSA at different temperatures and exposure times. Modified BI-BSA has a strong negative association with log IgG antibody titre levels (r = -0.65, P = 0.000) that was (-0.25, P = 0114) for sIgA in both the diabetic and control group, whereas IgG and sIgA log antibody titre levels has the strongest association with mBI-BSA heat processed at 70ºC/5 min in both groups (r = -0.78 and -0.77, P = 0.000, respectively). The logistic regression analysis yielded that IgG and sIgA log antibody titre levels produced to untreated BSA, mBI-BSA and heat processed mBI-BSA in both DC and NDC were associated with a relatively similar risk of developing diabetes.

Table (1): Multivariate analysis of tests between-subjects; effects for native Bovine Serum Albumin (BSA) and modified and heat processed BSA with bovine insulin (mBI-BSA) on serum IgG and secretory IgA (sIgA) titre levels in both diabetic and non-diabetic Jordanian children aged 14 years or less. Data were considered statistically significant at P<0.05.

Between-antigens effects	F	P-Value
Anti-BSA sIgA and anti mBI-BSA IgG	5.211	0.029
Anti-mBI-BSA sIgA and anti-mBI-BSA IgG (60 ºC / 5 min)	4.836	0.035
Anti-mBI-BSA (50 ºC / 5 min) sIgA and anti-mBI-BSA IgG (50 ºC / 5 min)	7.028	0.013
Anti-mBI-BSA (60 ºC / 5 min) sIgA and anti-mBI-BSA IgG (60 ºC / 15 min)	4.511	0.042

DISCUSSION

To the best of the researchers' knowledge, this study constitutes the first study on the immune response in young Jordanian DC aged 14 years or less with regard to their breastfeeding and early feeding practices. Worldwide, this study is also the first to develop a new saliva-based ELISA to determine the salivary IgA antibodies to dietary proteins particularly bovine milk

proteins in diabetic children.

Saliva collection is a non-invasive, stress-free, simple method, and can be easily collected and stored (James-Ellison et al., 1997). Therefore, sIgA has been widely used as a biomarker in a number of fields. 1) biobehavioural studies (Ben-Aryeh et al., 1990; Shirtcliff et al., 2001); 2) toxins and pollutants (Bauer et al., 1983); 3) as diagnostic tool (Ciclitira and Ellis, 1991); and 4) to determine the levels of antibodies produced to gliadin in patients with celiac autoimmune disease (Fasano et al., 2003).

Salivary IgA antibodies are one dominant characteristic humoral factor of the local immune system in the body secretions; therefore, it can act as first-defence line against local infections as well as preventing the access of foreign antigenic factors to the immune system (Roitt et al., 2001). The high synthesis rate of the T cells dependent sIgA antibodies and the direct association with foreign factors as well as half-life and direct association with the activation of both T and B cells (Smith et al., 1987; Paul, 1993) have made sIgA a better immune marker over measuring B and T cells in blood serum (Miletic et al., 1996).

Therefore, stabilisation of the mucosal barrier may become an important factor in gut health maintenance (Kleessen et al., 2003) that may affect gut permeability to possible foreign, dietary and non-dietary antigens and impair secretory immunity. Substantial interactions with other ingredients in food systems may result in modifying the behaviour of proteins (Kinsella and Whitehead, 1989).

Bovine serum albumin particularly ABBOS peptide (Karjalainen et al., 1992; Saukkonen et al., 1994) and insulin and proinsulin (B: 9-23 peptide) (Wegmann and Eisenbarth, 2000; Nakayama et al., 2005) are among the most putative environmental dietary proteins implicated in setting of beta cell destruction. Insulin is one of the most strongly implicated as a potential primary autoantigen capable of initiating the destruction of insulin secreting beta cells sanctioning other pancreatic proteins to become the primary target leading to the disease. It has also been anticipated that insulin becomes a target ensuing the initiation of the autoimmune process by another autoantigen (Alleva et al., 2000; Nakayama et al., 2005).

Bovine insulin is a small hapten and cannot elicit an immune response by itself. For insulin to initiate an immune response, it requires coupling to a carrier protein such as BSA (Harlow and Lane, 1988; Carter and Ho, 1994). The immunogenicity of antigens can often be profoundly altered by slight changes in the physical nature of the antigen; many molecules can be made more immunogenic by denaturation.

The immunogenicity of antigens can often be profoundly altered by slight changes in the physical and chemical nature of the antigen; many molecules can be made more immunogenic by denaturation. Given that aggregate-free antigens may inhibit the immune responsiveness (Kletter et al., 1971; Harlow and Lane, 1988; Saukkonen et al., 1994), protein antigens aggregate caused by heat processing are usually more immunogenic. Heat treatment changes the structure of many compounds particularly proteins and expose new epitopes. This modification can either alter the regions of the immunogen to present better sites for T-cell binding or expose new epitopes for B-cell binding. While antibodies can differentiate between conformations of protein antigens, small structural changes of the epitope can prevent the recognition of the antigen. Antibodies can differentiate between conformations of protein antigens (Harlow and Lane, 1988).

Substantial interactions with other ingredients in food systems may result in the modified behaviour of the

proteins. Compared to unmodified BSA, bovine serum albumin modified with bovine insulin has showed significant changes in spectral and chromatographic profiles; similar changes have been observed in response to various heating methods, various temperatures and exposure times (Al-Domi et al., 2004; Al-Domi et al., 2005).

On the surface, it would appear to be a logical association with the risk or protection from diabetes, nevertheless evidence on the use of breastfeeding or early infant feeding restrictions to prevent or minimise the disease occurrence is of limited strength and should not be misinterpreted by parents and the public (Atkinson and Gale, 2003). Human retrospective studies remain discrepant and controversial and are featured by a number of factors. First, differences between low and high risk populations examined (Esfarjani et al., 2001). Second , the actual exposure to certain dietary and non-dietary diabetogenic factors (Norris et al., 2003). Third, the timing of exposure to triggering factors (Kimpimaki et al., 2001). Fourth, ethical implications particularly in preventive trials (Rosenbloom et al., 2000). Finally, the design and analysis methods used in studies (Karjalainen et al., 1992).

CONCLUSION

The saliva-based ELISA system may be a useful proxy of immune responses and may constitute new grounds for the ongoing dietary intervention trials. The modification and thermal processing procedure of BSA is a merely sophisticated system in eliciting immune responses and is neither limited to diabetic patients nor associated with breastfeeding or early infant feeding practices, and may reflect unspecific defect of the immune system. Variation between low and high-risk populations requires further investigation.

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تطوير نظام ELISA جديد لتحديد الاستجابة المناعية ضد بروتينات الحليب عند الأطفال الأردنيين المصابين بسكري النوع الأول: افتقار العلاقة بين ظهور المرض وأنماط تغذية الرضع

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ملخـص

يتزايد الاهتمام عالمياً بتحديد العوامل البيئية المحتملة التي قد تؤدي إلى إثارة عملية المناعة الذاتية في خلايا البنكرياس عند الأشخاص الذين عندهم استعداد وراثي. تمت دراسة الاستجابة المناعية ضد بروتين مصل دم الأبقار (BSA)، وضد BSA المعدل بإنسولين الأبقار (mB1-BSA) وذلك قبل المعالجة الحرارية وبعدها. تكونت عينة الدراسة من خمسين طفلاً أردنياً مصابين بسكري النوع الأول أعمارهم 14 سنة أو أقل، ومثلهم من غير المصابين شكلوا مجموعة الدراسة الضابطة. جمعت عينات الدم واللعاب، وحددت مستويات المعيار الحجمي للأجسام المضادة (Secretory Iga (sIgA), IgS) المنتجة ضد كل من BSA، mB1-BSA الطبيعيين والمعالجين حرارياً، كذلك جمعت البيانات حول أنماط تغذية عينة الدراسة بما في ذلك الرضاعة الطبيعية باستخدام استبانة طورت سابقاً. إن أعلى معدل تغيير في مستويات المعيار الحجمي للأجسام المضادة (sIgA, IgG) المنتجة ضد mB1-BSA المعالج حرارياً (70°م / 5 دقائق). وقد ظهر في mB1-BSA غير المعالج حرارياً أيضاً معدل تغير نسبي كبير في مستويات المعيار الحجمي للأجسام المضادة مقارنة بتلك المنتجة ضد BSA غير المعالج حرارياً.وارتفع متوسط لوغاريثم تأثير mB1-BSA الطبيعي على مستويات المعيار الحجمي للأجسام المضادة عند المصابين وغير المصابين بنحو 1.3 ضعف مقارنة بـ BSA الطبيعي (sIgA; p =0.000, IgG; p =0.114) وارتفع كذلك متوسط لوغاريثم تأثير mB1-BSA المعالج حرارياً (70°م / 5 دقائق) على مستويات المعيار الحجمي للأجسام المضادة (sIgA, IgG) عند المصابين وغير المصابين بنحو 1.5 ضعف مقارنة بـ BSA غير المعالج (p =0.000) وبنحو 1.1 ضعف مقارنة بـ (mB1-BSA) غير المعالج حرارياً (p =0.000). ولم يلاحظ وجود استجابة مناعية ضد mB1-BSA المعالج حرارياً على درجات حرارة 70°م / 10 دقائق، 80°م / 5 دقائق، 60°م / 5 دقائق. إن نظام ELISA المعتمد على اللعاب قد يكون النظام المناسب لدراسة الاستجابة المناعية، مما قد يشكل قاعدة جديدة لدراسات التدخلات الغذائية القائمة. إن تعديل مصل دم الأبقار ومعالجته حرارياً لا يمكن إلا أن يكون نظاماً معقداً في إثارة الاستجابة المناعية ولكنه غير مقصور على المصابين بسكري النوع الأول أو غير المصابين بالمرض مما قد يعكس خللا غير محدد في الجهاز المناعي.

الكلمات الدالة: مناعة ذاتية، تفاعل مناعي، ممارسة غذائية، نضوج الأمعاء، تعديل البروتين، حرارة، السكري.

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