Investigating Immune Responses to Putative Diabetogenic Modified Bovine Proteins Subjected to Microwave Heating among Young Jordanian Children with Type 1 Diabetes Mellitus

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Abstarct: The objective of this study was to examine the effect of household Microwave Heating (MWH) on the immunogenicity of a putative diabetogenic protein among young Jordanian diabetic children. Bovine serum albumin (1 mg mL^{-1}) was modified at physiological pH (7.4) by incubating with equal volume of bovine insulin (1 mg mL^{-1}) at 37°C for 24 h. The developed modified Bovine Serum Albumin with Bovine Insulin (mBI-BSA) was subjected to MWH for 30, 60, 90 and 120 sec, cooled immediately to 0°C prior to analysis. Blood samples were collected from 76 diabetic and healthy children. Sera were tested for presence of antibodies against mBI-BSA by ELISA. Anti-BSA, anti-mBI-BSA and anti-microwave heated mBI-BSA antibodies were detected in diabetic and healthy children. The difference between groups was insignificant (p<0.05). Anti-mBI-BSA antibodies were significantly higher than that produced against native BSA (p>0.05) with no significant difference between groups. The difference in sera and saliva titers against mBI-BSA between diabetic children and their control was insignificant (p>0.05).The effect of MWH with different exposure times on anti-mBI-BSA was nearly similar to that produced against un heated mBI with no significant difference between groups.

Key words: BSA, insulin, immunogenicity, diabetes, autoimmune, protein

INTRODUCTION

Type 1 diabetes mellitus is a chronic metabolic organ-specific progressive immune-mediated disease resulting in detrimental effects on all parts of the body inducing degenerative complications in various organs particularly the insulin secreting pancreatic cells (Verge *et al.*, 1994). However, none of the potential primary determinants of immune-mediated type 1 diabetes mellitus has been unequivocally established (Akerblom *et al.*, 2005; Nakayama *et al.*, 2005; Kent *et al.*, 2005; Kimpimaki *et al.*, 2001; Lefebvre *et al.*, 2006). Although, Bovine Insulin (BI) is a small protein (hapten) and cannot elicit immune response by it self, it has been implicated as a putative primary autoantigen in animals (Nakayama *et al.*, 2005; Atkinson *et al.*, 1986; Srikanta *et al.*, 1986; Komulainen *et al.*, 1999; Kimpimaki *et al.*, 2002; Harfenist and Craig, 1952; Sanger, 1958). Rather it requires a linkage to a large immunogenic

carrier such as Bovine Serum Albumin (BSA) (Roitt *et al.*, 2001; Carter and Ho, 1994; Harlow and Lane, 1999). The modification of either BSA or bovine insulin (Teale and Benjamin, 1976; Speth and Lee, 1984) with different binding molecules as well as exposure to heat processing could have a substantial effect on their immunogenic response (Hanson and Mansson, 1961; Strand, 1994; Alting *et al.*, 1997; Karjalainen *et al.*, 1992). The modification as well as heat processing of BSA with Bovine Insulin (mBSA-BI) at various critical heating temperatures and exposure times to various heat processing methods (water bath and direct hot plate) has triggered different secretory and humoral immune responses with substantial variation from that of native compounds (Al-Domi *et al.*, 2008a).

Microwave, heat treatment has been widely used since, the 1940s in both large-scale food industry and household functions including infant formula or food preparation due to its high speed and convenience

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(Mermelstein, 1997; Campanone and Zaritzky, 2005). Microwave heating cause quick temperature rise in a food material by generating heat energy inside the foodstuff without requiring a medium as vehicle for heat transfer (Campanone and Zaritzky, 2005). Studies have demonstrated that the effect of microwave heating on both thermo-oxidative stability of lipids used in domestic applications and on both macro and micro nutrients as well as sensory attributes was substantial (Vikram *et al.*, 2005; Let *et al.*, 2005; O'Keefe *et al.*, 1994; Turkkan *et al.*, 2008; Verardo *et al.*, 2009). However, findings of refolding experiments on enhanced folding and denaturation of certain proteins by non-thermal effect of microwave heating remain inconsistent.

This is the 1st report on studying the effect of microwave heating on the immunogenicity of certain bovine milk proteins such as BSA or the newly developed putative diabetogenic protein (mBI-BSA) in young diabetic Jordanian children aged ≤14 years by using a newly developed saliva-based Enzyme Linked Immunosorbent System (ELISA) (Al-Domi *et al.*, 2008a).

MATERIALS AND METHODS

Design and setting: A cross-sectional study was conducted during July 2005-2008 to determine the presence of sera and saliva specific antibodies against microwave heat treated BSA modified with Bovine Insulin (mBI-BSA) using a saliva-based Enzyme Linked Immunosorbent Assay (ELISA) in young Jordanians diagnosed with type 1 diabetes mellitus aged ≤14 years.

The population sample: The population sample size study consisted of 76 Jordanian children with type 1 diabetes mellitus aged ≤14 years. To ensure representation, participants were recruited randomly from teaching, public and private hospitals and primary health care centers in Jordan.

About 76 unrelated, age and sex matched Non-Diabetic Children (NDC) were recruited from primary health care centers. The diagnosis of diabetes was confirmed based on the presence of clinical symptoms such as hyperglycemia, ketonuria, polyuria and polydepsia as well as the need for insulin therapy right after diagnosis according to the WHO diagnosis criteria. Control participants were unrelated to those with diabetes or to each other.

Ethical approval: 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. Participants or their parents were informed briefly about the nature and objectives of the study and asked to take part in the study and a verbal or written consent were obtained.

Collection of blood samples: About 76 fasting venous blood samples (10 mL) were drawn from all participants and controls by a licensed phlebotomist by venipuncture into a clot (red top) test tube. Whole blood samples were stored overnight at 4°C and then centrifuged at 4°C for 15 min at 1000× g. Sera was stored frozen at -20°C following the procedure of Jonstone and Thrope (1996).

Collection of saliva samples: About 76 saliva samples were collected from diabetic and their age and sex matched controls. Participants with periodontal diseases, respiratory infections or those undertaken major dental work were excluded. Whole unstimulated saliva samples were collected at least 30 min after the last meal or drink to minimize the potential of saliva contamination particularly from dairy products; participants were instructed to rinse their mouth thoroughly with water 10 min prior to collecting the saliva samples. Children were seated comfortably (Miletic *et al.*, 1996) and encouraged to relax and visualize one of their favorite foodstuffs.

Saliva samples (2-5 mL) were drawn by either passive slavering into a sterile swab tube or by a sterile plastic transfer suction pipette. Saliva samples were clarified in a refrigerated centrifugation for 15 min at 112× g (Castro *et al.*, 2004). To break down mucopoly-saccharides, saliva samples were exposed to a single freeze-thaw cycle and were aliquated and kept frozen at -20°C until used (Shirtcliff *et al.*, 2000; Worthman *et al.*, 1990).

Preparation of the testing antigens: Bovine serum albumin and bovine insulin (Sigma-Aldrich MO, USA) were reconstituted in ultrafiltered water (0.18 µm) over 24 h. Bovine serum albumin (1mg mL) was modified at physiological pH (7.4) by incubating with equal volume of BI (1 mg mL^{-1}) at 37°C for 24 h under strictly aseptic physiological conditions. A household microwave oven was used for heating the testing antigen samples. About 2 sterile-filtered native BSA and mBI-BSA samples (10 mL each) were placed in a 50 mL sterile beaker and were microwaved for 30, 60, 90 or 120 sec at a frequency of 2450 MHz and a power output of 1000 watts on a rotary turnable microwave plate. Heat processed bovine proteins were removed, cooled immediately to 0°C and samples were stored at -20°C for 24 h prior to analysis.

Measurement of antibody titres in saliva and serum: Enzyme-Linked Immunosorbent Assay (ELISA) was used to determine anti-human IgG and sIgA tire levels as described earlier by Al-Domi *et al.* (2008b). Briefly, multiwell, high-binding flat-bottom microtitre plates

(Greiner-Bio-One GmbH, Germany) were coated with native BSA (1 µg well⁻¹), native mBI-BSA (1 µg well⁻¹) or mBI-BSA (1 µg well⁻¹) subjected to microwave heating (30, 60, 90 and 120 sec) prepared in 0.1 M phosphate-buffered saline (PBS, pH 7.4). Plates were incubated at 4°C for 12 h. After washing (0.05% Tween 20 in 0.1M PBS), plates were blocked with 1% thermally treated normal sheep serum (DakoCytomation, Denmark) and incubated for 1 h at 37°C. After another washing step, sera and saliva were added in 1:200 dilution in PBS containing 1% thermally treated normal sheep serum, 0.05% Tween 20 and the plates were sealed and incubated for 1 h at 37°C. Following several washings, a 100 µL well⁻¹ of a mixture of rabbit anti-human IgG specific for γ and α -chains alkaline-phosphatase-conjugated (Dako Cytomation, Denmark) were added at 1:3000 diluted in 0.05 Tween 20 in 0.1M PBS and plates were incubated for 1 h at 37°C. Plates were developed by adding 100 µL well⁻¹ p-nitrophenylphosphate substrate (Sigma-Aldrich, St. Lois, MO) and incubated for 6 h at 37°C.

The reaction was stopped by adding 100 µL well⁻¹ 1M NaOH and the end-point was measured with microplate reader (ELX 800, Bio-Tek Instruments Inc.; Kcjunior software) at 405 nm. Optical density values were corrected with the OD values measured in uncoated wells. Duplicates varying by >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 OD value *2. Sera and saliva titres were expressed as the 50% of the reciprocal dilution factor of OD just above the cut-off point. Titers less than or equal to the cut-off point were assumed zero. Results were expressed as optical density units.

Statistical analysis: The differences between groups were analysed by Chi-square (χ^2) for categorical and 2-tailed Mann-Whitney U-test for continuous variables. All p>0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Serum and saliva levels of antibodies produced against native BSA, native BSA modified with Bovine Insulin and mBI-BSA subjected to microwave heating at various exposure times (30, 60, 90 and 120 sec) were compared in the children with type 1 diabetes mellitus and healthy children without any known family history of diabetes mellitus. Anti-BSA, anti-mBI-BSA and anti-microwave heated mBI-BSA antibodies were detected in all participating children with type 1 diabetes as well as

in healthy children. Although, higher in children with diabetes, IgG and sIgA titre levels produced against native BSA were not statistically significant between groups (p = 0.157). Whereas, sera and secretory anti-native mBI-BSA antibodies were increased significantly in all children compared to that produced against native BSA (p<0.05) but there was no significant difference between diabetic children and their control in respect of different antibody levels to mBI-BSA (Fig. 1).

Sera and secretary antibody titre levels produced against microwave heated mBI-BSA were almost similar to that produced against native mBI-BSA with slight variation but insignificant (p = 0.24) for all exposure times with no significant different between diabetic and health children. Nevertheless, antibodies produced against mBI-BSA microwaved for 30, 60, 90 and 120 sec were significantly higher than that produced against native BSA (p = 0.05) with no significant difference between the groups. Although, anti-BSA, anti-mBi-BSA and microwave heated mBI-BSA titre levels were slightly higher in children with type 1 diabetes than those in healthy children, antibody titre levels did not show any association with disease status in this study.

Figure 2 delineates percent change in the sera IgG and sIgA antibody titre levels produced against both native and microwave heated mBI-BSA with reference to IgG and sIgA antibody titre levels in both diabetic and healthy children produced in response to unheated BSA
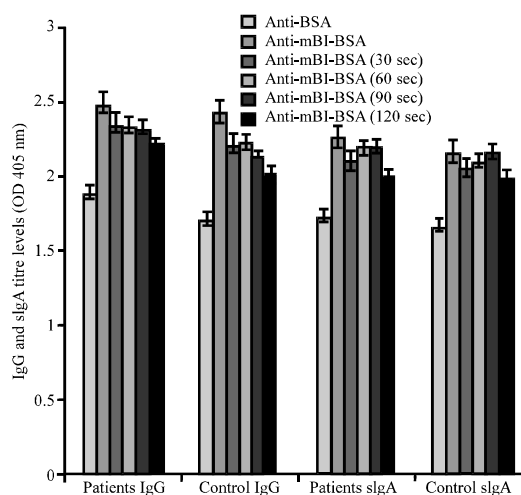

Fig. 1: Anti-BSA, anti-mBI-BSA and antiBI-BSA subjected to microwave heating at 30, 60, 90 and 120 sec mean IgG and secretory IgA (sIgA) antibodies titre levels in children with type 1 diabetes mellitus and healthy children. Data is considered significantly different at p<0.05; Mann-Whitney U-test
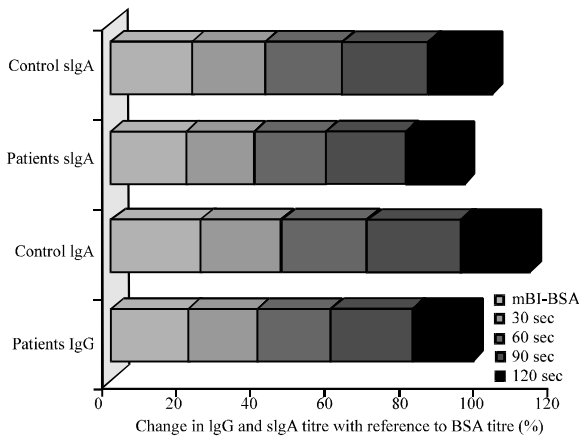
Fig. 2: Percent change in mean IgG and secretory IgA (sIgA) titre levels produced against mBI-BSA, and mBI-BSA subjected to microwave heating (30, 60, 90 and 120 sec) with reference to antibodies produced against native BSA among Jordanian children aged ≤14 year diagnosed with type 1 diabetes. Data is considered significantly different at p<0.05

(Fig. 1). Results show that the percent change in IgG and sIgA titre levels produced against native mBI-BSA was increased by approximately 25% in both children with type 1 diabetes and healthy children. Obviously, compared to anti-BSA antibodies, the percent change in anti-mBI-BSA subjected to 30, 60, 90 and 120 sec of microwave heating has declined by approximately 16-18%. The effect of exposure times on the variation in percent change in the effect of exposure times on antibodies produced against microwave-heated mBI-BSA was consistence and similar to that produced against native mBI-BSA with slight variation. Nevertheless, there was no significant difference between the groups in respect to percent change in antibody levels. This study to the best of the knowledge is the first study in which levels of sera and saliva antibodies produced against a newly developed putative immunogenic; Bovine Serum Albumin modified with Bovine Insulin (mBI-BSA) subjected to microwave heating at various exposure times (30, 60, 90 and 120 sec) were measured in a cohort of diabetic and healthy children. The findings of this study are consistent with our earlier findings (Al-Domi *et al.*, 2008b) in which both studies provide evidence for the presence of anti-BSA antibodies in both children with type 1 diabetes and healthy children.

The amount of IgG antibodies to BSA in its native conformation was about the same in both the sera and saliva of the children in both in diabetic and control with no significant difference between them.

The apparent lack of significant difference in antibody titers between both diabetic and control is not clear yet. Nevertheless, it may imply that these antibodies are either have no role in the autoimmune process against the insulin producing pancreatic β-cells or it could be a secondary phenomenon that resulted from the damage of β-cells (Couper *et al.*, 1999). Numerous physiological consequences are caused by adverse reactions to food that can lead to either immunologically or non-immunologically mediated reactions resulting in a wide range of signs and symptoms (Miller, 1998). The majority of people have high circulating levels of antibodies produced against a number of common microorganisms with high immunogenic antigens (Miller, 1998) such as viruses. Some of these microbial antigens are shared with antigens in milk proteins (Miller, 1998; Vela, 1997).

While, circulating antibodies to food proteins are also common in the general population (Kletter *et al.*, 1971), antibody titres to bovine milk proteins are usually lower and less common in adults than in children (Kletter *et al.*, 1971). The development of a state of unresponsiveness to dietary proteins such as bovine serum albumin in adults can be a result of prolonged minimal antigenic stimulation (Korenblat *et al.*, 1968).

Interestingly, the findings of the current study present additional evidence for the presence of anti-mBI-BSA antibodies in both children with type 1 diabetes and their healthy controls (Al-Domi *et al.*, 2008a) but with no significant difference between them. This finding indicates that the conformation of the BSA was different from the BSA modified with the bovine insulin. Nevertheless, anti-mBI-BSA IgG and sIgA antibody titre levels were significantly different those titres of anti-BSA IgG and sIgA antibodies in both diabetic and health children (p<0.05) with no significant difference between both groups. Bovine insulin, a small hapten (5,808 Daltons), differs from human insulin only by three amino acid residues (Yip *et al.*, 1998; Harfenist and Craig, 1952; Sanger, 1958). It cannot elicit an immune response by itself and therefore to initiate an immune response, it requires coupling to a carrier protein such as BSA (66430.3 Daltons) (Harlow and Lane, 1988; Carter and Ho, 1994). Both modification and thermal treatment of native proteins cause changes to the native conformation resulting in the formation of new epitopes. These epitopes elicit a different immune response from those of the native BSA molecule.

These findings are verified by earlier studies signifying that modification of both BSA (Teale and Benjamin, 1976) and bovine insulin with various binding molecule as well as heat processing have a substantial

effect on their immunogenic response (Hanson and Mansson, 1961; Alting *et al.*, 1997; Karjalainen *et al.*, 1992; Al-Domi *et al.*, 2008b).

The results in this study appeared different from findings by Al-Domi *et al.* (2004, 2005, 2008b) who then reported a significant difference between antibodies produced against mBI-BSA exposed to water bath heating compared to that produced against both BSA or unheated mBI-BSA (Al-Domi *et al.*, 2004, 2005, 2008b). The present results demonstrated that the amounts of IgG and sIgA antibodies produced in response to mBI-BSA subjected to microwave heating at various exposure times were almost similar to that produced in response to unheated mBI-BSA with no significant difference between the patients and controls. Nevertheless, the microwave heated mBI-BSA antibodies were significantly higher than that produced against BSA. Percent change in IgG and sIgA titre levels was increased by approximately 25% in both diabetic and healthy children compared to that produced against unconjugated BSA.

Bovine serum albumin exposed to 70°C 5 min^{-1} water bath heating has shown the substantial relative change in the both IgG and sIgA titre levels whereas unheated mBI-BSA has also shown a significant relative change in antibody titre levels compared to that of untreated BSA (Al-Domi *et al.*, 2008b).

Heat treatment changes the antigenicity of proteins by denaturing epitopes while exposing others (Harlow and Lane, 1988). Variations in the percent change in antibody titre levels produced against microwave heated mBI-BSA provide further evidence linking various heating methods (water bath, ohmic, infrared and microwave) as well as temperatures and exposure times to changes in immunogenicity of proteins including dietary proteins. Nevertheless, the different heating sources will exert their different impact based on mechanism of heating (Vikram *et al.*, 2005; Al-Domi *et al.*, 2004, 2008b). Microwave energy for instance, penetrates food materials and generates a volumetrically distributed heat source, due to molecular friction caused by dipolar rotation of polar solvents and the conductive migration of dissolved ions. As a result, microwave heating cause quick temperature rise in a food material provided that microwaves have the capacity to generate heat energy inside the foodstuff without requiring a medium as vehicle for heat transfer (Campanone and Zaritzky, 2005).

It seems that household microwave ovens were not capable of causing significant changes in the physical nature of mBI-BSA as inferred from our results. However, the specific mechanisms responsible for the observed variation in the effect of the heating methods as well as exposure times still need to be further examined. The microwave heat treatment in this study failed to develop any significant changes in the immunogenicity of the BSA, B-BSA separately or complex as reflected from the insignificant difference in results obtained using sera or saliva of diabetic patients or the control (Shazman *et al.*, 2007; Bohr and Bohr, 2000). Modern infant formulae require both large scale and home food processing which can bring about extreme alteration in the chemical and physical nature of food components (Rechcigl, 1982). Household preparation methods of infant foods and formulas vary between the application of severe heat processing temperature and extended boiling times to a less severe heat processing temperature and shorter exposure times such as the application of microwave fields (Singh *et al.*, 1998).

Recent findings by Akerblom *et al.* (2005) pointed to certain modifications in dietary ingredients during formula processing that might lead to the development of pancreatic autoimmune disease and consequently diabetes.

Remarkably, the findings of this study are consistent with the earlier findings (Al-Domi *et al.*, 2008b) which have indicated that immune responses to both BSA mBI-BSA and subjected to different heating methods were neither limited to diabetic patients nor associated with the breastfeeding or early infant feeding practices. We have even demonstrated that a higher proportion of diabetic children received no bottle-feeding in their early infancy reflecting an unspecific defect of the immune system in young Jordanian children (Al-Domi *et al.*, 2008b). Nevertheless further large-scale studies are required to support and substantiate the findings of the studies. Variation between low and high-risk populations requires further investigation. Immune responses were neither limited to diabetic patients nor associated with the breastfeeding or early infant feeding practices (Al-Domi *et al.*, 2008a). Substantial interactions with other ingredients in food systems may result in the modified behaviour of the proteins (Kinsella and Whitehead, 1989). Changes in mucosal architecture and concomitant interactions between different dietary (Speth and Lee, 1984; Teale and Benjamin, 1976) and non-dietary constituents such as viral infections or effect of normal flora (Sharma *et al.*, 1995; Clarke, 1975) coexisting in the gut environment can either boost or suppress the autoimmune process (Scott *et al.*, 1996) may consequently cause such deleterious effect on the pancreatic cells.

CONCLUSION

In this study, 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 minimize the disease occurrence is of limited strength and should not be misinterpreted by parents and the public. Nevertheless, it was evident that modification of certain dietary proteins as well as using different heating methods and exposure times prompted substantial changes in the antibodies titre levels with no significant differences between children with type 1 diabetes and healthy children.

Further, studies are required to determine specific and sensitive immune responses caused by modification and heat treatment of probably other dietary proteins that might be implicated in the pathogenesis of type 1 diabetes.

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