DNA Damage and Neutrophil Elastase in Children with Prader-Willi Syndrome

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Obesity is the most common cause of metabolic problems in Prader-Willi syndrome (PWS). Obesity has been joined to a low grade pro-inflammatory state, in which impairments in the oxidative stress and antioxidant mechanism could be involved. The aim of the work is to investigate the level of DNA damage and inflammatory marker neutrophil elastase in PWS patients. The study included 21 children with PWS detected by fluorescence in situ hybridization (FISH) method and 20 age and sex healthy matched obese controls. Their mean age was 6 ± 2.24 years. Leukocyte DNA damage was evaluated by comet assay and neutrophil elastase was assessed by ELISA. All patients presented with distinctive faces, hypotonia, obesity, short stature and various other criteria. FISH revealed deletion 15q11–13 in all PWS patients. The mean of DNA damage frequency was significantly higher in PWS than controls. The body fat%, body mass index (BMI) z score were elevated in PWS cases. Moreover, the neutrophil elastase was significantly higher in patients compared to controls. The present study highlights the existence of oxidative stress and inflammation in Prader Willi syndrome that may have a role in the management and treatment of these patients.

Keywords: Comet Assay Prader Willi syndrome; DNA damage; Neutrophil Elastase; BMI.

Prader, Willi, and others described Prader-Willi syndrome (PWS) in 1956, and more than 1500 subjects have now been reported in the medical literature.

Prader-Willi syndrome (PWS; 176270) is a complex genetic disorder that affects many parts of the body. The absence of expression of the paternal genes from the imprinted chromosomal region 15q11.2—q13 is the cause of PWS syndrome1,2. PWS is distinguished by hypotonia (94% of subjects), childhood obesity (94%), deficiency in mental status with average IQ of 65, ranged from 20 to 90 (97%), shortness in stature (76%), small hands and feet (83%), hypogenitalism/hypogonadism (95%), and a typical face with narrow bi-frontal diameter, almond-shaped eyes and a triangular mouth1,4. The occurrence of PWS is expected to be about one in
10,000 to 25,000 live births and is considered the most frequent syndrome cause of marked human obesity. Hyperphagia, persistent hunger, decreased perception of satiety and an uncontrollable appetite with impaired emesis are thought to cause obesity.

Oxidative stress is considered one of several adverse cellular responses to obesity. It can then damage cellular structures and triggers a low grade pro-inflammatory state. Association between adipose tissue inflammation and insulin resistance is considered as an important risk factor in the etiology. Immune cells such as macrophages, T–cells, B–cells, mast cells and eosinophils have all been involved in this process. Neutrophils are the first immune cells which respond to inflammation and can promote a more chronic inflammatory state by helping to recruit macrophages and interacting with antigen presenting cells. Neutrophil elastase (NE) is one of several proteases which is secreted from neutrophils and it can endorse inflammatory responses in several diseases. Recent studies have suggested that obesity is associated with chronic adipose tissue inflammation, which results in increased levels of proinflammatory factors, such as neutrophil elastase.

This study aimed to investigate whether PWS is associated with oxidative stress and inflammatory marker neutrophil elastase.

**MATERIALS AND METHODS**

We studied 82 obese patients, 42 revealed the clinical picture of PWS and 21 were confirmed as deleted by FISH.

The mean age of PWS cases with genetic confirmation was ±2.24 years and age ranged from 4 to 17 years, 10 males and 11 females. For comparison, 20 healthy age and sex matched obese controls were recruited. PWS were referred to the outpatient clinic of the Pediatric Department, Faculty of Medicine, Benha University. Written informed consent was obtained from all the patients.

Exclusion criteria for all participants included type 1 diabetes, presence of metabolic disease (confirmed by use of drugs), pulmonary diseases and sepsis.

All participants were subjected to thorough medical history and clinical examination, and anthropometric measurements were performed as follows: height was measured to the nearest 0.5 cm; body weight was measured to the nearest 0.1 kg; body mass index (BMI) was calculated as weight/height^2 (kg/m^2). Z weight and ZBMI were calculated according to WHO Anthro Plus program. Body composition was carried out using a body composition analyzer TANITA SC–330 (Tanita Corporation, Tokyo, Japan). BF% was estimated to the nearest 0.1%.

Laboratory diagnosis of deleted type of PWS is achieved by FISH performance according to the modification of 11 and manufacturer instructions using locus-specific probe (LSI) Prader-Willi SNRPN (15q11) supplied by Kreatech Diagnostics (United Kingdom). Twenty one patients diagnosed by FISH, that had deletion as the etiology of the disease were included in this study.

The level of serum neutrophil elastase was assessed by ELISA, supplied by Immunodiagnostic AG Stubenwald-Allee 8 Ad-64625 Bensheims. Sample size of 21 and 20 achieve 100% power to detect a difference of neutrophil elastase 6.5 between the null hypothesis that both groups means are 15.9 and the alternative hypothesis that means of group 2 is 9.4 with estimated group standard deviations of 5.0 and 2.4 and with a significance level (alpha) of 0.05 using a two-sided two-sample t-test. DNA damage in leukocytes was performed and estimated according to Palus et al., 1999.

**Measurement of Comet Assay**

**Cell preparation**

Peripheral blood leukocytes were isolated by centrifugation (30 min at 1300g) in Ficoll-Paque density gradient (Pharmacia LKB Biotechnology, Piscataway, NJ, USA). After centrifugation, leukocytes in the buffy coat were aspirated and washed twice by phosphate-buffered saline at pH 7.4 (PBS).

**Preparation of cell microgels on slides**

The comet assay was performed according to with modifications according to. Cell microgels were prepared as layers. The first layer of gel was made by applying 100 μl of normal melting point agarose (0.7%) onto a precleaned microscope charged slides and cover slipped gently. The coverslip was removed after the agarose solidified at 4°C. Low melting-point agarose
(0.5%) was prepared in 100 mmol/L PBS and kept at 37°C. Mononuclear cells were mixed with the low melting-point agarose and 100 µl of the mixture was applied to the first gel layer. The slides were then covered with a coverslip and placed at 4°C for solidification. After the second layer solidified, the coverslips were removed from the cell microgels. A final layer of low-melting agarose was added followed by coverslips, left to solidify for 10 minutes then the coverslips were removed.

Table 1. Clinical and demographic data of PWS patients.

<table>
<thead>
<tr>
<th>Clinical Picture</th>
<th>DM</th>
<th>FH</th>
<th>Cons</th>
<th>Age/sex</th>
<th>Case</th>
</tr>
</thead>
<tbody>
<tr>
<td>Round face, micropenis, hypotonia</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>7/M</td>
<td>1</td>
</tr>
<tr>
<td>Broad bossing forehead, full cheeks, absent labia minora, mild ID, hypotonia</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>13/F</td>
<td>2</td>
</tr>
<tr>
<td>Broad bossing forehead, full cheeks, hypoplastic clitoris, hypotonia</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>17/F</td>
<td>3</td>
</tr>
<tr>
<td>Almond eye, broad bossing forehead, full cheeks, hyperpigmentation, hypotonia, absent labia minora and clitoris</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>9/F</td>
<td>4</td>
</tr>
<tr>
<td>Broad bossing forehead, large ears, short fingers, hypotonia, clinodactyly, café au lait patches</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>11/M</td>
<td>5</td>
</tr>
<tr>
<td>Microcephaly, undescended testis, micropenis, moderate ID, hypotonia</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7/M</td>
<td>6</td>
</tr>
<tr>
<td>Broad bossing forehead, hypospadias, mild ID, hypotonia</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>10/M</td>
<td>7</td>
</tr>
<tr>
<td>Broad bossing forehead, micropenis, moderate ID, hypotonia</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>12/M</td>
<td>8</td>
</tr>
<tr>
<td>Hypospadias, hyperpigmentation, nocturnal enuresis, mild ID, hypotonia</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>7/M</td>
<td>9</td>
</tr>
<tr>
<td>Broad bossing forehead, hypoplastic clitoris, mild ID, hypotonia</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>15/F</td>
<td>10</td>
</tr>
<tr>
<td>Broad bossing forehead, short fingers, absent labia minora, moderate ID, hypotonia</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>10/F</td>
<td>11</td>
</tr>
<tr>
<td>Full cheeks, nystagmus, moderate ID, hypotonia</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>7/F</td>
<td>12</td>
</tr>
<tr>
<td>Broad bossing forehead, micropenis, hepatosplenomegaly, mild ID, hypotonia</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11/M</td>
<td>13</td>
</tr>
<tr>
<td>Almond eyes, broad bossing forehead, full cheeks, epicanthal folds, syndactyly, simian crease, acanthosis negricans, hypotonia, hypoplastic labia minora, absent clitoris</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>9/F</td>
<td>14</td>
</tr>
<tr>
<td>Broad bossing forehead, absent labia minora, hepatosplenomegaly, nystagmus, hypotonia</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>7/F</td>
<td>15</td>
</tr>
<tr>
<td>Broad bossing forehead, bulbus nose, hypospadias, mild ID, hypotonia</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>8/M</td>
<td>16</td>
</tr>
<tr>
<td>Broad bossing forehead, moderate ID, hypotonia</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>10/M</td>
<td>17</td>
</tr>
<tr>
<td>Full cheeks, hepatosplenomegaly, hypotonia</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>5/F</td>
<td>18</td>
</tr>
<tr>
<td>Broad bossing forehead, hypospadias, mild ID, hypotonia</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>6/M</td>
<td>19</td>
</tr>
<tr>
<td>Almond eyes, broad bossing forehead, full cheeks, epicanthal folds, hypotonia, hypoplastic labia minora, moderate ID</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>9/F</td>
<td>20</td>
</tr>
<tr>
<td>Full cheeks, hepatosplenomegaly, hypotonia</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>4/F</td>
<td>21</td>
</tr>
</tbody>
</table>

Cons=Consanguinity; FH=Family history; DM=dysmorphism
Lysis of cells, DNA unwinding, gel electrophoresis, DNA staining

The slides were covered with 100 ml of fresh lysis buffer at pH 10 at 4°C for 1h. Buffer contains: 2.5 mol/L NaCl, 100 mmol/L EDTA, 1% sodium hydroxide, 10 mmol/L Tris, 1% Triton X-100, 10% (Dimethylsulfoxide) DMSO. After draining, microgels slides were treated with DNA unwinding solution (300 mmol/L NaOH, 1 mmol/L EDTA, pH 13) for 30 min at 4°C, and placed directly into a horizontal gel electrophoresis chamber filled with DNA-unwinding solution. Gels were run with constant current (300 mA at 4°C) for 30 min. After electrophoresis, the microgels were neutralized with 0.4 M Trisma base at pH 7.5 for 10 min. The slides were stained with 20 µl ethidium bromide (10 µg/ml).

Visualization and analysis of Comet Slides

The slides were examined at 400× magnification using a fluorescence microscope (Leica Microsystems, CMS GM b H, Wetzlar, Germany. Model DM 2500), equipped with an excitation filter of 549 nm and a barrier filter of 590 nm. A damaged cell is visualized as each cell had the appearance of a comet, with a brightly fluorescent head and a tail to one side formed by the DNA containing strand breaks that were drawn away during electrophoresis. Samples were analyzed by counting the damaged cell out of 100 cells per slide to calculate the percent of damage.

Written informed consent was obtained from the parents after the explanation of the aim of the study. All the patients’ data were confidential, neither the data nor the collected samples were used in any other research.

Statistical analysis

The statistical analysis was conducted with the Statistical Package for Social Sciences (SPSS version 17. Initially, the normality of the data using the Kolmogorov-Smirnov test was tested. Data are presented as mean ± standard deviation.

Mann–Whitney U test has been used to compare comet assay results between the PWS cases and controls. While, Student t-test was applied for comparisons of continuous variables as the anthropometric and biochemical parameters. P values less than 0.05 were considered to be statistically significant for each test.

RESULTS

Out of the 21 diagnosed patients with Prader Willi syndrome, fourteen patients (66.6%) were the offspring of consanguineous marriage. Dysmorphic features were present in 10 patients including round face, broad bossing forehead, almond eye. Hypotonia was present in all patients. Abdominal and pelvic ultrasonography showed hepatosplenomegaly in 4 patients. Genitalia showed absent labia minora and or hypoplastic clitoris in 7 females and micropenis or hypospadias in 6 males. Intellectual disability was present in

Fig. 1. Damaged DNA in leukocytes of PWS. It shows slightly damaged DNA.

Fig. 2. Damaged DNA in leukocytes in PWS. It shows a severely damaged DNA.
13 patients ranging from mild to moderate ID (Table 1).

FISH revealed deletion 15q11–q13 in 21 patients. Z weight was 3.7±2.1, BMI z score was 5.3±1.9 and body fat % was 44.5±15.9 (Table 2).

Levels of neutrophil elastase and DNA damage in Prader Willi patients were higher compared to controls (Table 3).

The DNA damage in the leukocytes of the patients (Fig. 1 and Fig. 2) was higher in patients than that of obese healthy controls (Fig. 3). The DNA was slightly damaged in some cases (Fig. 1), while it was more severe in others (Fig. 2). Fig. 4 shows image of FISH, 46,XX,del(15)(q11.2).ish del(15)(q11.2)(SNRPN-).

### DISCUSSION

PWS occurs equally in males and females and in all ethnic group although disproportionately more Caucasians are reported17–19. This is consistent with our study as we had 10 males and 11 females.

Fourteen patients were the off springs of consanguineous marriage. High incidence of consanguinity was noticed in this study.
(66.6%) as in our population consanguinity is high about 60% \(^ {20-22} \). Dysmorphic features were present in 10 patients including round face, broad bossing forehead, almond eye. All patients in our study showed hypotonia which is a basic diagnostic criterion for PWS patients that might be due to the decrease of physical activity and depression\(^ {21} \). In meta-analysis of PWS the behavioral features associated with PWS has not been fully investigated. Significance difference in clinical features of cognitive development and psychiatric illness are associated with different molecular defects of PWS\(^ {24} \).

Abdominal and pelvic ultrasonography showed hepatosplenomegaly in 4 patients may be due to the extreme obesity and associated liver enzyme dysfunctions. Genitalia showed absent labia minora and or hypoplastic clitoris in 7 females and micropenis or hypospadias in 6 males. Intellectual disability was present in 13 patients ranging from mild to moderate ID. These findings are consistent with previous reports who reported that clinical features include hypotonia, hypogonadism, short stature, intellectual disability, and obesity\(^ {3,25} \). Our results show that Z weight and Z BMI were high in PWS patients. Also, body fat % was elevated in PWS cases. This findings in agreement with previous study\(^ {26} \)noted that the main cause of morbidity and mortality in PWS patients is obesity.

Excessive appetite and overeating and lack of sexual development are some of the symptoms of PWS\(^ {27} \). In comparison with obese subjects, those with PWS had lower energy expenditure and decreased lean body mass. Previous study reported that the energy expenditure of at rest in PWS was significantly suppressed by 16% compared to the obese subjects\(^ {28} \).

PWS is a complex phenomenon caused by disturbance in the hypothalamic satiety regulatory mechanisms contributed by several hormones, body composition differences, low physical activity, altered feeding behavior and increased dietary intake\(^ {29} \).

PWS represents the most common form of genetic obesity; one of the most concerning symptoms is hyperphagia. Obesity is a major cause of increased morbidity and mortality in patients with PWS\(^ {29,30} \). The present study is the first one which examined the relationship between serum elastase and DNA damage in Prader Willi patients. This study found that serum elastase level and DNA damage were significantly higher in Prader Willi patients as compared to age and sex matched healthy controls.

Huang et al. 2017 highlighted that inflammation plays an important role in the development of obesity-related complications. Extensive investigation has demonstrated the relationship between obesity and increased systemic inflammation\(^ {7,32} \). Subsequently, neutrophils are the most abundant leukocytes that are critical for innate immunity and acute inflammation and neutrophil elastase is considered a secretagogue for cytokines and a modulator of inflammation\(^ {7} \).

Neutrophils play a critical role in protecting the host against microbial pathogens, but they produce proteolytic enzymes that can destroy tissue and lead to organ failure such as NE that cause lung injury and respiratory failure via pulmonary vascular and alveolar damage\(^ {33} \).

El-Eshmawy et al. 2011 suggested that neutrophils play an essential role in obesity-related inflammation. Furthermore, other study\(^ {34} \)elucidated that elastase may be used as a non-specific indicator to screen inflammation and infection. Obesity is also associated with metabolic dysfunction that contributes to higher incidence of inflammation-associated chronic disease and greater susceptibility to infection\(^ {35} \).

Currently, it is widely accepted that oxidative stress (OS) is involved in the pathogenesis of PWS\(^ {36} \). We verified the presence of OS by estimating the DNA damage in the leukocytes of the patients which was higher than that of controls. Previous studies have already exhibited a relationship between obesity and oxidative stress\(^ {37} \). Antioxidants such as vitamin A, E and C could improve oxidative stress and consequently the low-grade inflammation. Our study proposed that obesity in PWS is associated with chronic low-grade inflammation, this coincides with the findings of Khan et al., 2018. Some studies\(^ {38} \)used medications such as Beloranib that improved inflammatory biomarkers. Antioxidants and anti-inflammatory drugs could improve the condition in these patients. Moreover, epigenetic-based therapy for the PWS has been reported recently\(^ {39} \).
CONCLUSION

The current study draws attention to the need of investigating the interplay between oxidative stress and inflammation with obesity in PWS.

REFERENCES


