OXIDATIVE STRESS IN PATIENTS WITH PHENYLKETONURIA

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Brazil
Phenylketonuria (PKU)

- IEM - 1:10.000 – 1:20.000

- 1934 – Asbjorn Folling:
  Increased urine excretion of phenylpyruvate that produced a green colour after ferric chloride reaction.
Phenylalanine hydroxylase

Deficiency

Phenylalanine

transaminase

Tyrosine hydroxylase

tyrosinase

melanin

Phenylpyruvate

catecholamines

Homogentisic acid

DOPA

Fumarate acetyl CoA

carbon dioxide

Phenyllactate

Phenylacetate
Patients with good adherence to treatment do not present the mental retardation characteristic of untreated PKU patients, although they can have a low IQ and neuropsychological deficits.

(Holtzman et al, 1986; Smith et al, 1990; Lou et al, 1985; Krause et al, 1985)
Phenylketonuria

Genetic Disease

Brain Damage

Neurologic Features

Mental Retardation
PKU – pathophysiology

- Inhibition of transport of neutral amino acids (val, leu, ileu, tryp, thre, hist, met, tyr) in the blood-brain barrier → defective myelination. (Curtis et al, 1981; Hanley et al, 2000)

- Tryptophan → serotonin
  Tyrosine → dopamine

- In vitro inhibition of pyruvate kinase by Phe → reduction of glucose uptake in the brain (Hasselbalch et al, 1996)

- Inhibition of creatine kinase in vivo and in vitro by Phe (Feksa et al, 2002)

- Decrease in the activity of succinate dehydrogenase in rat cortex with hyperphenylalaninemia and in vitro inhibition of complexes I-III of the respiratory chain by Phe (Rech et al, 2002)

- In cultured neurons, Phe inhibits glutamatergic synaptic transmission (Glushakov et al, 2003).
PKU Neurophysiopathology

High Phe levels

Oxidative Stress

Free radicals

Free radical: species that contains one or more unpaired electrons.

Reactive oxygen species (ROS): $\mathrm{O}_2^{*-}$, $\mathrm{H}_2\mathrm{O}_2$, $\mathrm{HOCl}$, $\mathrm{ONOO}^-$, $\mathrm{NO}^*$, $\mathrm{OH}^*$

(Halliwell and Gutteridge, 2007)
Oxidative stress

**Antioxidant defenses:**

- Enzymatic: SOD, CAT, GPx
- Non-enzymatic: endogenous and exogenous substances

**Consequences:**

- Oxidative damage to lipids, proteins and DNA

(Dröge, 2002; Halliwell and Gutteridge, 2007)
CNS – High vulnerability to Oxidative Stress

- high oxygen consumption
- high metabolism: ROS generation
- high iron and lipid contents (polyunsaturated fatty acids)
- low activity of antioxidant defenses

(Halliwell and Gutteridge, 2001)
Oxidative Stress – IEM (other than PKU)

- Propionic and Methylmalonic Acidemia
  (Fontella et al, 2000)

- Glutaric Acidemia Type I
  (Latini et al, 2002)

- Tyrosinemia Type I (Bird et al, 1995)

- MSUD (Barschak et al, 2006)

- X-ALD (Vargas et al, 2004)
Oxidative Stress – IEM

1. accumulation of toxic metabolites that lead to excessive production of free radicals

2. metabolic by-products directly or indirectly deplete the cell antioxidant capacity

3. restricted diets alter the antioxidant status
Evidence of Oxidative Stress in PKU Patients
Antioxidant Defences in PKU Patients

Reduction of selenium in plasma and urine of children with phenylketonuria during treatment with low protein diet:

<table>
<thead>
<tr>
<th></th>
<th>Chromium</th>
<th>Selenium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasma</td>
<td>Urine</td>
</tr>
<tr>
<td></td>
<td>(n = 19,19)</td>
<td>(n = 14,15)</td>
</tr>
<tr>
<td>PKU children</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\bar{x} \pm SD$</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Median</td>
<td>11.5</td>
<td>&lt;9.1</td>
</tr>
<tr>
<td>Range</td>
<td>&lt;9.6–53.8</td>
<td>&lt;3.9–11.7</td>
</tr>
<tr>
<td>Siblings</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\bar{x} \pm SD$</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Median</td>
<td>&lt;9.6</td>
<td>&lt;7.1</td>
</tr>
<tr>
<td>Range</td>
<td>&lt;9.6–30.8</td>
<td>&lt;2.7–15.1</td>
</tr>
</tbody>
</table>

Antioxidant Defences in PKU Patients

Decreased GPx activity

(Wilke et al, 1992; Reilly et al, 1990; van Bakel et al, 2000; Darling et al, 1992; Sierra et al, 1998)
Antioxidant Defences in PKU Patients

Decreased activity of GPx x selenium deficiency:

(Wilke et al, 1992; Reilly et al, 1990; van Bakel et al, 2000; Darling et al, 1992)
Antioxidant Defences in PKU Patients

Selenium supplementation does not correct the GPx activity?

- Problems in the absorption of selenium?
- Poor dietary compliance?

(Sierra et al, 1998)

(Lambruschini et al, 2005)
Antioxidant Defences in PKU Patients

Selenium deficiency x cognitive changes:

(Castaño et al, 1997; Gassio et al, 2008)

Figure 1. Plasma selenium concentrations in PKU patients with normal and altered CPT performance. Selenium deficiency was considered for values lower than 50 µg/L. CPT = Conner’s Continuous Performance Test.
Antioxidant Defences in PKU Patients

Decrease of GPx and neurological manifestations

<table>
<thead>
<tr>
<th></th>
<th>BP</th>
<th>NE</th>
<th>IQ</th>
<th>EEG</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKU normal GSH-Px</td>
<td>32%</td>
<td>27%</td>
<td>13%</td>
<td>14%</td>
</tr>
<tr>
<td>PKU low GSH-Px</td>
<td>65%</td>
<td>39%</td>
<td>29%</td>
<td>30%</td>
</tr>
</tbody>
</table>

Results are expressed as percent of patients with neuropsychological disturbances.

BP: behavioral problems
NE: neurological examination
IQ: intellectual quotient

(Sierra et al, 1998)
Antioxidant Defences in PKU Patients

Reduction of ubiquinone (coenzyme Q10) in the plasma of PKU patients:

- Q10: lipophilic antioxidant
- Low protein diet?
- Inhibiting the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase by Phe, decreasing the biosynthesis of Q10?

Control group: 0.69 µ mol / L
Group 1: well-controlled patients
Group 2: patients with poorly controlled

Antioxidant Defences in PKU Patients

Decreased antioxidant status (TAS and TAR):

![Graph showing decreased TAR levels in PKU patients]

**TABLE 1**
Plasma antioxidant variables in patients and control subjects

<table>
<thead>
<tr>
<th></th>
<th>PKU patients (n = 24)</th>
<th>HPA patients (n = 10)</th>
<th>Control subjects (n = 42)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selenium (μmol/L)</td>
<td>0.52 ± 0.20</td>
<td>0.92 ± 0.21</td>
<td>1.23 ± 0.14</td>
</tr>
<tr>
<td>α-Tocopherol (μmol/L)</td>
<td>21.46 ± 4.06</td>
<td>19.25 ± 2.11</td>
<td>20.93 ± 6.15</td>
</tr>
<tr>
<td>Uric acid (μmol/L)</td>
<td>217.9 ± 50.4</td>
<td>235.7 ± 27.8</td>
<td>244.4 ± 104.8</td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>39.8 ± 2.2</td>
<td>40.5 ± 2.3</td>
<td>39.5 ± 3.2</td>
</tr>
<tr>
<td>TAS (mmol/L)</td>
<td>1.52 ± 0.14</td>
<td>1.52 ± 0.13</td>
<td>1.76 ± 0.19</td>
</tr>
</tbody>
</table>

*μ ± SD. PKU, phenylketonuria; HPA, hyperphenylalaninemia; TAS, total antioxidant status.*

(Van Bakel et al, 2000; Sitta et al, 2006; Sitta et al, 2009; Sirtori et al, 2005)

low levels of selenium
Antioxidant Defences in PKU Patients

Decrease of GSH in erythrocytes of PKU patients

Table 3

<table>
<thead>
<tr>
<th></th>
<th>PKU patients (n = 24)</th>
<th>HPA patients (n = 10)</th>
<th>Control subjects (n = 42)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH (μmol/g Hb)</td>
<td>4.71 ± 0.64²</td>
<td>6.17 ± 1.30</td>
<td>7.35 ± 2.32</td>
</tr>
<tr>
<td>Glutathione peroxidase (U/g Hb)</td>
<td>27.75 ± 12.30⁶⁴</td>
<td>49.59 ± 11.99</td>
<td>48.78 ± 7.46</td>
</tr>
<tr>
<td>SOD (U/mg Hb)</td>
<td>1.49 ± 0.55³</td>
<td>1.57 ± 0.36²</td>
<td>2.40 ± 0.33</td>
</tr>
<tr>
<td>Glutathione reductase (U/g Hb)</td>
<td>9.17 ± 1.72</td>
<td>9.67 ± 2.59</td>
<td>9.01 ± 2.01</td>
</tr>
<tr>
<td>Glutathione transferase (U/g Hb)</td>
<td>4.22 ± 1.69</td>
<td>4.11 ± 0.86</td>
<td>4.66 ± 1.50</td>
</tr>
</tbody>
</table>

Group A: Early diagnosis
Group B: late diagnosis

- Inhibition of transport system for Gly and Cys by high levels of Phe?
- Increased formation of free radicals?

(van Bakel et al, 2000; Sitta et al, 2009)
Antioxidant Defences in PKU Patients

Correlation between selenium and GSH

<table>
<thead>
<tr>
<th></th>
<th>Selenium</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>P</td>
<td></td>
</tr>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Tocopherol (μmol/L)</td>
<td>−0.05</td>
<td>0.78</td>
<td></td>
</tr>
<tr>
<td>Albumin (g/L)</td>
<td>0.33</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>Uric acid (μmol/L)</td>
<td>0.37</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>TAS (mmol/L)</td>
<td>0.18</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>FT₄ (pmol/L)</td>
<td>−0.46</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>FT₃ (pmol/L)</td>
<td>0.27</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>FT₄:FT₃</td>
<td>−0.55</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>rT₃ (pmol/L)</td>
<td>−0.56</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>rT₃:FT₃</td>
<td>−0.59</td>
<td>&lt;0.0005</td>
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</tr>
<tr>
<td><strong>Erythrocyte</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione peroxidase (U/g Hb)</td>
<td>0.76</td>
<td>&lt;0.000001</td>
<td></td>
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<td>GSH (μmol/g Hb)</td>
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<td>&lt;0.05</td>
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<td>0.54</td>
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<td>0.79</td>
<td></td>
</tr>
<tr>
<td>Glutathione reductase (U/g Hb)</td>
<td>−0.06</td>
<td>0.74</td>
<td></td>
</tr>
</tbody>
</table>

(van Bakel et al, 2000; Sitta et al, 2009)
Antioxidant Defences in PKU Patients

Decrease of enzymatic antioxidant defenses (SOD and CAT):

TABLE 3
Antioxidant enzyme activities of the glutathione cycle and reduced glutathione in erythrocytes of patients and control subjects

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</tr>
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</table>

(van Bakel et al, 2000; Artuch et al, 2004; Gassió et al, 2008)
Antioxidant Defences in PKU Patients

Decreased plasma levels of L-carnitine in patients with good adherence to the PKU diet

(Sitta et al, 2009)
Antioxidant Defences in PKU Patients

- Negative correlation between TBARS and carnitine.
- Positive correlation between TAR and carnitine.

L-carnitine:
- Main sources: red meat and dairy.
- Antioxidant and antiperoxidative

(Sitta et al, 2009)
Oxidative Biomarkers in PKU Patients

Lipid peroxidation

Diagnosis
(late diagnosis)
(Sirtori et al, 2005)

Treatment
(late diagnosis)
(Sitta et al, 2006)

Group I: Phe between 5.2 to 9.4 mg / dL
Group II: Phe between 17.3 to 21.1 mg / dL
Oxidative Biomarkers in PKU Patients

Lipid peroxidation

Group A: Early diagnosis
Group B: Late diagnosis

(Sitta et al, 2009)
Oxidative Biomarkers in PKU Patients

**Lipid peroxidation**

<table>
<thead>
<tr>
<th></th>
<th>All (n = 58)</th>
<th>Group 1 (n = 25)</th>
<th>Group 2 (n = 33)</th>
<th>HPA group (n = 30)</th>
<th>Control group (n = 58)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phe (µmol/L)</strong></td>
<td>604 (152–1407)&lt;sup&gt;2,3&lt;/sup&gt;</td>
<td>607 (210–1244)&lt;sup&gt;2,3&lt;/sup&gt;</td>
<td>601 (152–1407)</td>
<td>255 (108–607)</td>
<td>50 (33–76)</td>
</tr>
<tr>
<td><strong>Q&lt;sub&gt;10&lt;/sub&gt; (µmol/L)</strong></td>
<td>0.55 (0.2–1.67)&lt;sup&gt;2,4&lt;/sup&gt;</td>
<td>0.37 (0.20–0.45)&lt;sup&gt;2,3,3&lt;/sup&gt;</td>
<td>0.57 (0.46–1.67)</td>
<td>0.63 (0.44–1.22)</td>
<td>0.71 (0.36–1.10)</td>
</tr>
<tr>
<td><strong>Tocopherol (µmol/L)</strong></td>
<td>20.2 (8.5–30.1)</td>
<td>17.8 (8.5–25.7)&lt;sup&gt;6,8&lt;/sup&gt;</td>
<td>21(13.0–30.1)</td>
<td>21.7 (15.1–30.0)</td>
<td>22.0 (12.0–36.0)</td>
</tr>
<tr>
<td><strong>Retinol (µmol/L)</strong></td>
<td>1.48 (0.6–3.6)</td>
<td>1.46 (0.6–3.1)</td>
<td>1.55 (0.8–3.6)</td>
<td>1.15 (0.8–1.8)</td>
<td>1.40 (0.6–2.3)</td>
</tr>
<tr>
<td><strong>Ascorbate (µmol/L)</strong></td>
<td>70 (36–109)</td>
<td>61 (44–97)</td>
<td>76 (36–109)</td>
<td>67 (38–89)</td>
<td>55 (8–92)</td>
</tr>
<tr>
<td><strong>Selenium (µg/L)</strong></td>
<td>49 (11.0–81.0)&lt;sup&gt;2,9&lt;/sup&gt;</td>
<td>45 (11.0–68)&lt;sup&gt;2,9&lt;/sup&gt;</td>
<td>49 (25–81)</td>
<td>60.6 (28.0–84.0)</td>
<td>65.4 (32–84)</td>
</tr>
<tr>
<td><strong>GPX (U/g Hb)</strong></td>
<td>19.8 (11.4–30.1)</td>
<td>20 (12–30.1)</td>
<td>19.7 (11.4–27.8)</td>
<td>20.4 (14.4–26.0)</td>
<td>21.8 (13.9–33.6)</td>
</tr>
<tr>
<td><strong>MDA (nmol/L)</strong></td>
<td>663 (316–1404)&lt;sup&gt;7&lt;/sup&gt;</td>
<td><strong>768 (497–1341)</strong>&lt;sup&gt;4,7,10&lt;/sup&gt;</td>
<td>620 (316–1404)</td>
<td>591 (357–946)</td>
<td>521 (320–914)</td>
</tr>
<tr>
<td><strong>Tocopherol intake (mg/d)</strong></td>
<td>13.1 (2.1–40.2)</td>
<td>12.3 (2.1–26.2)</td>
<td>13.8 (5.8–40.2)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Retinol intake (µg/d)</strong></td>
<td>1622 (434–5806)</td>
<td>1767 (434–5806)</td>
<td>1523 (480–4674)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Ascorbate intake (mg/d)</strong></td>
<td>112 (9–327)</td>
<td>124 (9–240)</td>
<td>104 (19–327)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Selenium intake (µg/d)</strong></td>
<td>40 (6–113)</td>
<td>39 (6–105)</td>
<td>43 (8–113)</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

(Colomé et al, 2003)
Oxidative Biomarkers in PKU Patients

Oxidative damage to proteins

Group A: Early diagnosis

Group B: late diagnosis

(Sitta et al, 2009)
Oxidative Biomarkers in PKU Patients

(Sitta et al, 2010)
Oxidative Biomarkers in PKU Patients

Oxidative DNA damage

**Group A:** patients with good adherence to treatment;

Phe = 156.6 ± 14.1 µmol/L

**Group B:** patients with poor adherence to treatment;

Phe = 1260.3 ± 32.8 µmol/L

**Group C:** Controls

(Schulpis et al, 2005)
Oxidative Biomarkers in PKU Patients

DNA damage in vitro and in vivo (comet assay)

Fig. 1. In vitro effect of phenylalanine on DNA damage (comet assay) in leukocytes from whole blood. Data represent median ± S.E. of 3 independent experiments (individuals). (a) p < 0.01 compared to the Phe 100 µmol/L group; (b) p < 0.01 compared to the 250 µmol/L group; (c) p < 0.01 compared to the Phe 500 µmol/L group (Kruskal–Wallis test followed by Mann–Whitney U-test).

Fig. 2. DNA damage (comet assay) of peripheral blood leukocytes from two groups of PKU patients, one with Phe < 600 µmol/L (n = 8) and the other with Phe > 600 µmol/L (n = 10) and controls (n = 17). Data represent median ± S.E. (a) p < 0.0001 compared to the control; (b) p < 0.001 compared to the Phe < 600 µmol/L group (Kruskal–Wallis test followed by Mann–Whitney U-test).

(Sitta et al, 2009)
Oxidative Stress in PKU – animals studies

Fernandes et al., 2010: in vitro effect of Phe on lipid peroxidation in rat hippocampus

Fernandes et al., 2010: in vitro effect of Phe on lipid peroxidation in rat cortex

EPG SYMPOSIUM - PKU 2011
Oxidative Stress in PKU – animals studies

- Fernandes et al., 2010: in vitro effect of Phe on glutathione levels in cortex and hippocampus of rats.

Fernandes et al., 2010: in vitro effect of Phe on oxidative damage to proteins in cortex and hippocampus of rats.

Exposure time: 60 min
Oxidative Stress in PKU – animals studies

Fernandes et al., 2010: In vitro effects of Phe metabolites on lipid peroxidation in rat cortex

PPA: phenylpyruvate
PLA: phenyllactate
PAA: phenylacetate

Exposure time: 60 min
Final Remarks

Oxidative Stress in PKU

Oxidative damage to proteins, lipids and DNA

Selenium
GSH

Coenzyme Q10
Carnitine

SOD
CAT
GPx

TAR
TAS
Final Conclusion

- It can be suggested that the oxidative stress is one of the pathomechanisms of neurological damage in PKU patients.
Final Conclusion

- Oxidative stress occurs at diagnosis and in treated PKU patients (good/bad compliance) demonstrating that:
  - oxidative damage occurs despite the dietetic treatment
  - dietary restrictions can contribute to depleted antioxidant status
  - Phe is not the only metabolite involved with oxidative events

EPG SYMPOSIUM - PKU 2011
Final Conclusion

- Studies on new therapeutic approaches concerning oxidative stress (antioxidants) will be beneficial to PKU patients.
Thank you for your attention!

Genetic Medical Service / HCPA-Brazil