Measuring serum oestradiol in women with Alzheimer's disease: the importance of the sensitivity of the assay method

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Abstract

Objective: Oestrogens could be protective against the development of Alzheimer's disease (AD) but reports on oestrogen levels in AD have been conflicting.

Design and methods: A meta-analysis using robust regression was carried out to assess whether the sensitivity of the assays of past studies had affected the reported level of total oestradiol. We had also measured total oestradiol in women with AD (n = 66) and controls (n = 62) not using hormone replacement therapy. We used two assays for total oestradiol to assess the difference between sensitive (radioimmunoassay with a specific rabbit antibody: 3 pmol/l) and relatively insensitive (immunoassay: 37 pmol/l) assays.

Results: Meta-analysis using robust regression indicated that insensitive assays gave higher levels of total oestradiol when many samples fall below the level of sensitivity of the method. Earlier reports of low levels of total oestradiol in AD might be explained by this phenomenon, since total oestradiol levels (using the sensitive assay) in our controls were one third of those reported in the earlier studies. Using the sensitive assay we found that women with AD had significantly (P < 0.01) higher levels (26 ± 13 pmol/l) of total oestradiol than controls (21 ± 13 pmol/l). Using the insensitive assay, there was no significant difference in the levels of total oestradiol.

Conclusions: The sensitivity of the assay determines the reported value of the oestradiol levels. Studies using a sensitive assay do not report significantly lower levels of total oestradiol in women with AD. This weighs against the hypothesis that low levels of total oestradiol are a risk factor for AD.

Introduction

Women have approximately twice the risk of developing Alzheimer’s disease (AD) compared with men (1). After the menopause, women have much lower levels of oestrogens than men, which could put them at risk for AD (2). Moreover, over the last 10 years, observational studies have consistently shown that women who take oestrogen replacement therapy (ORT) after the menopause have a lower risk of developing AD and possibly other types of dementia (3, 4). In theory, ORT should protect against age-related cognitive decline and dementia (5). Indirect experimental evidence supports this proposition. Oestrogens, such as oestradiol, have shown many neuroprotective effects in in vivo and in vitro animal experiments (6).

In spite of the evidence outlined above, evidence that ORT prevents age-related cognitive decline and dementia is inconclusive. First, it is difficult to assess the direction of causality in epidemiological studies that have investigated the risk reduction for AD with ORT use (4). For instance, women who decided to take ORT after the menopause were more healthy prior to taking ORT, which would decrease their risk for AD regardless of the use of ORT (7). Secondly, three randomised controlled trials did not show any positive effect of oral ORT in women with AD (8–10). Thirdly, elderly men actually have greater cognitive decline than women (11), despite having higher levels of oestrogens (12, 13). Lastly, observational studies comparing oestrogen levels in women with AD and controls have found inconsistent results. Two studies found lower oestrogen levels in AD (14, 15), two found higher levels in AD (16, 17) and five found no significant differences (18–22). The reasons for the discrepancies between observational studies of oestrogen levels in dementia are unclear. A remarkable difference was that the overall levels of sex steroids reported differed between studies. For example, total oestradiol levels differed by more than a factor of four between some studies. Surprisingly, this difference was greater for control groups than for cases (4). One possible
reason for this result is that some studies used insensitive assays and classified immeasurable values as missing, so that they then obtained unrealistically high average hormone levels (14, 16). The difference in assay sensitivity is known to affect the average value of total oestradiol (23). We tested this possibility in two ways. We earlier reported higher oestradiol levels (but not of oestrone or testosterone) using a very sensitive assay in AD cases, compared with controls, which were independent of confounds, such as age, sex, hormone binding globulin, body-mass index (BMI), blood pressure, smoking, APOE genotype and diabetes (24). In the present study, first we compared the results of two total oestradiol assays, one a very sensitive method using a highly specific rabbit antibody (25) and the other a less sensitive standard immunoassay used in the hospital’s clinical biochemistry laboratory. Secondly, we carried out a meta-analysis of the published data to see whether the sensitivity of the assay method might explain the discrepancies.

Subjects and methods

Subjects

All subjects included were women not using hormone replacement therapy: 62 controls without objective memory impairment and 66 AD cases according to the National Institute of Neurological and Communicative Disorders and Stroke, and the Alzheimer’s Disease and Related Disorders Association (NINCDS/ADRDA) criteria (26) from the Oxford Project To Investigate Memory and Ageing (OPTIMA). Thirty-seven of the AD cases fulfilled the Consortium to Establish a Registry for Alzheimer’s Disease (CERAD) histopathological criteria for probable or definite AD (27). Controls, patients and their closest relatives had all given informed consent prior to the study, which had local ethics committee approval. All participants had undergone medical examination, which included blood sampling, brain scans (CT or MRI and SPECT) and cognitive assessment using the Cambridge Examination for Mental Disorders of the Elderly (CAMDEX (28)). None of the participants had active disease or was institutionalised.

Oestradiol assays

We obtained non-fasting blood serum samples between 1000 and 1200 h and then stored these at −70 °C for an average of 2.3 ±2.1 years. For total oestradiol, we used two assays. For the first technique, duplicate serum samples were extracted with ether. Total oestradiol was then assessed by radioimmunoassay using a highly specific polyclonal rabbit-derived anti-human oestradiol antiserum. The lower limit or sensitivity of this assay was set at 3 pmol/l. The between assay coefficient of variation (CV) was 13% (for a mean total oestradiol level of 27 pmol/l), the overall within assay CV was 9% (4% for a mean total oestradiol level of 14 pmol/l). Cross-reaction with oestrone was low (0.03%). The validity of this assay when compared with other commercial assays (such as Diagnostic Products Corporation (DPC) C-A-C, UK) was high (r > 0.99) and was assessed in a study of parallelism in samples. The data were linearised using a 2-parameter log-logit transformation. The standard curve and all samples gave r^2 > 0.99. The standard curve had a slope of −1.78 and the samples had a mean slope of −1.61±0.4025. The second total oestradiol assay we used was the commercial Bayer Immuno 1 assay (Bayer, Bayer Corporation, Tarrytown, NY, USA) carried out according to the manufacturer’s instructions. The lower detection limit for this commercial assay was 37 pmol/l (and results below this limit are considered unreliable), but the expected values for postmenopausal women are between 0 to 172 pmol/l. Consequently, when using this technique, 80% of the data for the women were missing. The correlation with a commercial kit (DPC C-A-C) was high (0.99, 1.02x+2.9 regression equation), in the range of 40–9000 pmol/l. At 110 pmol/l, the total CV was 10% and the within runs CV was 8% (n = 114). At 268 pmol/l, these coefficients of variation were reported to be 5% and 4% respectively. The between assay correlation (between the sensitive ether extraction immunoassay, ‘x’, and the less sensitive Bayer, ‘y’, immunoassay) was r = 0.90, P < 0.005 (n = 157) and the overall regression equation was y = 0.81x+1.13. The data using the sensitive total oestradiol assay have been shown in a previous report (24).

Statistics

We used t-test in SPSS (version 10.0 for Windows (29)) to test the two different oestradiol assays. We also investigated why the total oestradiol levels differed between studies using Pearson’s correlations and robust regression analyses (30). For all analyses, the level of significance was set at 0.05.

Results

Table 1 shows the clinical characteristics of the cases and the control groups.

Results of the different total oestradiol assays

Using the highly specific and sensitive assay technique, total oestradiol levels were higher in AD cases than controls, both unadjusted and adjusted for age and both natural and log transformed (Table 1). We also had total oestradiol data for 107 subjects using a less sensitive assay (where the limit was set at 37 pmol/l, instead of 3 pmol/l). Results of the two assays (including
the data under the specified sensitivity level and the data of the hormone users) corresponded quite well with one another. The slope (1.046) was not significantly different from 1 \( (t = 31.09, P < 0.001) \) and the intercept (constant = 1.925) was not significantly different from zero \( (t = 0.58, P = 0.56) \). The correspondence of the assays was also reflected in the average mean levels (Table 1). Again AD cases had significantly higher levels than controls. However, if we excluded participants who had total oestradiol levels lower than 37 pmol/l (and thus treating them as missing values), the average total oestradiol levels of AD cases did not differ and were, in fact, non-significantly lower than those of controls. It should be noted that only a few subjects (only 18\%) actually had oestradiol levels above this cut-off and remained in these analyses (Fig. 1).

**Meta-analyses of other studies investigating levels of total oestradiol in AD and controls**

We investigated why the total oestradiol levels differed so much between studies, using robust regression. These analyses included nine studies (Table 2) which provided data on total oestradiol levels and assay sensitivity (9, 15–20, 22, 31). The mean ages (between 72 and 80 years, with the number of studies included in the analysis \( k = 9 \) ) and BMIs \( k = 4 \) did not correlate with total oestradiol levels in AD cases or controls (both \( P > 0.40 \)) over all studies. The storage time of the blood samples (which varied from a number of months to 7 years, \( k = 8 \)) or the time of serum collection (e.g. morning only or all day) were also not associated with total oestradiol levels over studies \( (P > 0.25, k = 6) \).

Robust regression indicated a significant linear association between assay sensitivity and total oestradiol levels, for both controls \( (b = 0.97[\text{sensitivity}]: \text{approximate 95\% confidence limits 0.018–0.028}) \) and AD patients \( (b = 0.55[\text{sensitivity}]: \text{approximate 95\% confidence limits 0.009–0.026}) \). This association is also clearly illustrated in Fig. 2. Robust regression also indicated that storage temperature related significantly to total oestradiol levels in controls \( (b = 0.72 \text{ per degree, approximate 95\% confidence limits 0.22–1.22}) \) and AD cases \( (b = 0.58 \text{ per degree, approximate 95\% confidence limits 0.32–0.84}) \). However, we are less confident that storage temperature determined oestradiol levels, since there were fewer published data available for analysis and some studies stored samples at different temperatures for varying times \( (15, 16) \).

**Discussion**

The most striking difference between our study and previous results related not to the total oestradiol levels of the AD patients (which were 29\% higher than those of controls), but to those of the controls,

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**Table 1 Clinical and biochemical characteristics of controls (CON) and Alzheimer’s disease (AD) cases.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>CON ((n = 62))</th>
<th>AD ((n = 66))</th>
<th>(P)-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical characteristics</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>76±8</td>
<td>77±8</td>
<td>0.64, ns</td>
</tr>
<tr>
<td>SHBG (nmol/l)</td>
<td>79±32</td>
<td>74±31</td>
<td>0.35, ns</td>
</tr>
<tr>
<td>BMI (weight/height(^2))</td>
<td>25±4</td>
<td>24±4</td>
<td>0.44, ns</td>
</tr>
<tr>
<td><strong>Biochemistry</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oestradiol (pmol/l) Very sensitive (3 pmol/l) ether-extraction radioimmunoassay</td>
<td>21±13</td>
<td>26±13</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Oestradiol (pmol/l) Less sensitive (37 pmol/l) Bayer immunoassay</td>
<td>22±14</td>
<td>34±32</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Oestradiol (pmol/l) Bayer assay using the 37 pmol/l cut-off</td>
<td>55±16 ((n = 5))</td>
<td>46±10 ((n = 14))</td>
<td>0.44, ns</td>
</tr>
</tbody>
</table>

* These \( P\)-values are derived from \( t\)-tests done between CON and AD cases. SHBG, sex hormone binding globulin; ns, not significant.

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![Figure 1 Distribution of total oestradiol levels (TE2 in pmol/l) of elderly women (66 AD and 62 controls aged between 57 to 99 years) not using hormone replacement therapy. The mean value was 23.6 pmol/l with an S.D. of 13.14 \((n = 128)\).](https://www.eje.org)
which in our study were less than a third of some of those in earlier studies (14, 15). In view of this result, and of the fact that our findings run counter to the expectation that sex steroids should have neuroprotective effects (5), we investigated why total oestradiol levels differed so much between studies.

The sensitivity of the assay seemed to be the main determinant of the reported levels of total oestradiol in previous studies. Studies that used less sensitive assays reported, on average, higher total oestradiol levels in AD. Our own data conformed, non-significantly, to this pattern. The only exception to this general rule was the study of Manly et al. (15) that reported high total oestradiol levels with an assay of high sensitivity (7 pmol/l). The upper limit of their total oestradiol range was 282 pmol/l. All our non-ORT users had total oestradiol levels below 80 pmol/l. This suggests that Manly et al. (15) may have included ORT users in their sample. Our study used a sensitive assay and low storage temperature, so the results may be more likely than those of previous studies to be valid. Several other studies (22, 32) found non-significant trends to higher total oestradiol levels in AD cases compared with controls. The fact that total oestradiol levels were significantly higher in AD cases in our study probably reflects, additionally, its greater power due to its larger size. Finally, our clinical assessment has shown high validity when compared with the post-mortem confirmed diagnosis of AD (33) and more than half the cases of the present study had post-mortem confirmation of Alzheimer pathology. This further increases our confidence in our own results: overall then, we conclude, in contrast to most previous studies, that oestradiol levels are higher in women with AD than in age-matched controls.

One explanation for this finding could be that the genotype of the oestradiol receptors (ER alpha and possibly beta) shows polymorphisms in AD cases (34–36). If ER are less sensitive in AD, this could result in a feedback deficiency which would increase luteinising hormone (LH) and follicle-stimulating hormone levels and, in turn, increase the conversion from oestrone to oestradiol. Increased levels of LH have been found in women with AD (37) and are suspected to have detrimental effects on the brain (38). However, our results do not provide an argument for causality: patients' higher levels of total oestradiol might have arisen as a result of AD rather than be one of the causes. Prospective studies are needed in subjects at risk of dementia to see whether raised levels of total oestradiol occur before the onset of the dementia. Nevertheless, our

Table 2 Oestradiol (E2) levels in AD cases and controls (CON), with age and BMI of participants, sensitivity of the assay and storage aspects.

<table>
<thead>
<tr>
<th>Study</th>
<th>n</th>
<th>Age (years)</th>
<th>E2 (pmol/l)</th>
<th>Sensitivity (cut-off assay) (pmol/l)</th>
<th>BMI (pmol/l)</th>
<th>Storage temperature (°C)</th>
<th>Storage time (years)</th>
<th>Time range sample collected*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manly et al. (15)</td>
<td>143</td>
<td>75</td>
<td>52</td>
<td>70.2</td>
<td>7</td>
<td>27</td>
<td>30</td>
<td>−20</td>
</tr>
<tr>
<td>Hogervorst et al. (17)</td>
<td>130</td>
<td>77</td>
<td>28</td>
<td>21</td>
<td>3</td>
<td>24</td>
<td>25</td>
<td>−70</td>
</tr>
<tr>
<td>Rasmussen et al. (22)</td>
<td>33</td>
<td>76</td>
<td>22</td>
<td>14</td>
<td>6</td>
<td>22</td>
<td>27</td>
<td>−70</td>
</tr>
<tr>
<td>Barrett-Conor &amp; Goodman-Gruen (18)</td>
<td>392</td>
<td>74</td>
<td>22</td>
<td>20</td>
<td>11</td>
<td>−70</td>
<td>7</td>
<td>7–11</td>
</tr>
<tr>
<td>Yaffe et al. (20)</td>
<td>425</td>
<td>72</td>
<td>24</td>
<td>18</td>
<td>90</td>
<td>6</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>Carlson et al. (19)</td>
<td>46</td>
<td>76</td>
<td>27</td>
<td>26</td>
<td>19</td>
<td>−50</td>
<td>0.1</td>
<td>12–13</td>
</tr>
<tr>
<td>Asthana et al. (31)</td>
<td>12</td>
<td>79</td>
<td>51</td>
<td>37</td>
<td>8</td>
<td>2</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>Cunningham et al. (16)</td>
<td>112</td>
<td>74</td>
<td>54</td>
<td>57</td>
<td>40</td>
<td>25</td>
<td>27</td>
<td>−20</td>
</tr>
<tr>
<td>Wang et al. (9)</td>
<td>47</td>
<td>72</td>
<td>24</td>
<td>100</td>
<td>73</td>
<td>7</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>Honjo et al. (21)</td>
<td>14</td>
<td>80</td>
<td>15</td>
<td>11</td>
<td>100</td>
<td>10</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>Fillit et al. (14)</td>
<td>32</td>
<td>78</td>
<td>45</td>
<td>91</td>
<td>100</td>
<td>15</td>
<td>1</td>
<td>15</td>
</tr>
</tbody>
</table>

* When the sample was collected during the day.

Figure 2 Levels of total oestradiol (pmol/l) in AD cases (open squares) and controls (open triangles) in different studies which clearly follow the cut-off for sensitivity of the assay (solid circles) and not the average age of the participants (solid squares). Man, Manly et al. (15), Hog, Hogervorst et al. (17), Ras, Rasmussen et al. (22), Bar, Barrett-Conor & Goodman-Gruen (18), Yaf, Yaffe et al. (20), Car, Carlson et al. (19), Ast, Asthana et al. (31), Cun, Cunningham et al. (16) and Wan, Wang et al. (9). The older studies of Hon, Honjo et al. (21) and Fil, Fillit et al. (14) did not report sensitivity levels although the latter reported that many data were missing (suggesting a low sensitivity).
findings are not consistent with the hypothesis that low levels of total oestradiol in women are a risk factor for AD.

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