SHORT COMMUNICATION

Stability of AMH measurement in blood and avoidance of proteolytic changes

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Richard Fleming, BSc PhD, is scientific director at Glasgow Centre for Reproductive Medicine and professor of reproductive medicine at the University of Glasgow, Scotland. He has worked in both research and clinical service, studying human ovarian function, for more than 30 years. He has been responsible for a number of innovative developments, including the first use of ultrasound to track follicular development (1979) and the use of gonadotrophin-releasing hormone agonists to control pituitary activity during ovarian stimulation (1982). Recent work on evaluation of ovarian reserve prior to assisted reproduction has led to an extended exploration of the understanding and clinical value of anti-Müllerian hormone through reproductive life.

Abstract The new Gen II assay for anti-Müllerian hormone (AMH) shows good stability and reliability in serum, but analyses of stability in whole blood are lacking. Testing the effects of storage of whole-blood samples at room temperature revealed significant increases in the measured value of AMH of 31% over 4 days ($P < 0.001$). The effect is temperature dependent, with storage at $4^\circ\text{C}$ showing markedly reduced increments. Further, samples collected into serum tubes with gel separators and centrifuged within 5 h (blood cells and serum physically separated within the collection tube) showed reliable stability over a period of more than 5 days.

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KEYWORDS: AMH, anti-Müllerian hormone, blood, concentration, serum, stability

Introduction

The value of estimating the concentration of anti-Müllerian hormone (AMH) in the circulation of young males and in women is due to its relationship with reproductive potential. Most clinical studies use one of two commercial assays: IOT (Immunotech, Marseille, France) DSL (Diagnostic Systems Limited, Texas, USA), which show proportionately different values (standardization) and sensitivities.

The AMH Gen II assay (Beckman Coulter, High Wycombe, UK) combines the calibrators of the IOT assay and the paired antibodies of the DSL assay, in a microplate-based ELISA format. It uses a pair of antibodies directed to epitopes in the mature region of AMH (Al-Qahtani et al., 2005) and is thought to be less affected by proteolysis of AMH in the sample. Examination of AMH stability using this assay demonstrated that AMH stability in serum was robust (Kumar et al., 2010). However, the demand for tests of AMH in women in centres lacking appropriate laboratory facilities has led to the use of remote laboratory services utilizing whole-blood dispatch and postal/delivery services. This can mean that blood samples are not processed within the recommended time scales. Furthermore, anecdotal reports

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suggest that AMH results in the same woman can show different values. These procedures have not been validated in the context of the Gen II AMH assay.

The aim of this study was to test the stability of AMH evaluations after exposure and storage in whole blood under various conditions. It became apparent that whole-blood storage influenced results directly and clarification was undertaken, as well as steps to reduce/avoid the problems were conducted.

Materials and methods

AMH assay

The assay used was the AMH Gen II ELISA assay. It was performed using the semi-automated programmed Evolis immunoassay system supplied by Bio-Rad Laboratories (Hemel Hempstead, UK). Kit instructions were followed and the AMH concentrations in the samples were interpolated from the calibration curve produced. The lower functional sensitivity of the assay had been previously established as 1.5 pmol/l (Wallace et al., 2011), so sample values below this were not included in the subsequent analyses.

Sample preparation and experimental steps

Blood samples were taken in duplicate for control (in red-top serum Vacutainer tubes; BD, ref 367837, 6ml) and experimental purposes, from women attending the GCRM fertility centre. The control sample was centrifuged immediately (1100 g for 10 min), refrigerated and assayed the following morning. It was also assayed simultaneously to the experimental sample. No specific ethical approval was sought, as samples were used anonymously.

Experimental sample handling

Whole-blood storage: effects of time and temperature

The experimental sample was stored at room temperature (20°C) for 20 h, 44 h and 90 h before centrifugation and assay. Only samples with T0 (control) evaluation above 1.5 pmol/l were evaluated (n = 87) in a series of 18 assays.

Physical separation of serum and blood cells

Samples were collected directly into gold-top Vacutainer tubes with gel separation (SST II Advance, no. 367954; BD). The samples were stored with the cells and serum separated by the gel plug as designed. The control samples were centrifuged within 5 h of collection and assayed the following morning, while the experimental samples were centrifuged simultaneously to the controls, but were then stored at room temperature for 5 days, with agitation. They were tested at 7 days, after a further 2 days refrigeration. This test was performed with 13 paired samples in total in three separate assay evaluations.

AMH assay

These examinations were effected in 18 assays during a period of 3 months with two internal quality control pooled serum samples: concentrations targeted at clinically relevant values (means of 4.1 pmol/l and 21.1 pmol/l) effected in all assays. The variations of these two pools were 16% and 13%, respectively, with no drift of the mean values during that period. This variability is higher than that reported by Kumar et al. (2010) and probably relates to the quality-control pools in this study being pooled plasma, whilst those in numerous studies, including Kumar et al. (2010), were a calibrator product supplied by the manufacturers. Tests were carried out using a sequence of kit supply lot numbers and samples were run as single evaluations. Individual comparison tests were always effected in the same assays.

Comparisons of sample concentrations at each time point were undertaken as paired t-tests. Progressive effects of time exposure (multiple time points) were examined using analysis of variance (ANOVA).

Results

Effects of storage time and temperature

Table 1 shows that when the sample was stored as whole blood at room temperature for an extended period of time, there was a clinically important increase in the estimated concentration of AMH of 31% over a delay of 3.5 days (about 90 h). The changes over time were significant (P < 0.001; ANOVA Kruskal–Wallis test). The incremental change was independent of the AMH concentration at T0.

When whole-blood samples were stored at 4°C for 90 h before serum separation, the increase in AMH was modest (approximately 10%) (Table 1).

Effect of physical separation of serum and blood cells

Table 2 shows that the mean AMH at T0 was 16.2 pmol/l and that there was negligible change upon retesting after 7 days (1%).

Table 1 Effect of time and temperature on AMH concentration in whole-blood samples.

<table>
<thead>
<tr>
<th></th>
<th>20 h at 20°C (n = 23)</th>
<th>44 h at 20°C (n = 11)</th>
<th>90 h at 20°C (n = 21)</th>
<th>90 h at 4°C (n = 32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control AMH (pmol/l)²</td>
<td>8.6</td>
<td>12.1</td>
<td>10.5</td>
<td>13.9</td>
</tr>
<tr>
<td>Experimental sample AMH (pmol/l)</td>
<td>9.9</td>
<td>13.6</td>
<td>13.8</td>
<td>15.4</td>
</tr>
<tr>
<td>Increment (%)</td>
<td>15</td>
<td>12</td>
<td>31</td>
<td>11</td>
</tr>
<tr>
<td>P (paired t-test)</td>
<td>&lt;0.001</td>
<td>&lt;0.016</td>
<td>&lt;0.001</td>
<td>0.002</td>
</tr>
</tbody>
</table>

²Control sample = serum immediately separated from blood cells and refrigerated until assayed within 24 h of venepuncture.
Discussion

It is clear from these results that protracted storage of whole blood at room temperature profoundly influenced the AMH concentration measured in the subsequently separated serum. This resulted in an incremental increase of approximately 31% over approximately 3.5 days at room temperature. The effect was time and temperature dependent. The increase at 20 h was modest (15%), which is just outwith the variation of the assay, and the effect was more substantial with increasing time (31% at 90 h), and these increases may be clinically important. The incremental increase was not so profound when whole blood was stored at 4°C (11%), suggesting that it is an enzymic phenomenon, requiring the presence of blood cells within the storage tube (i.e. direct contact of blood cells and serum). This was confirmed by the negligible change in the measured value (1%) seen in separated serum samples, despite prolonged storage at 20°C. These phenomena were similar in plasma (data not shown). These results have implications for postal/courier-based assay services.

When samples were collected into gold-top Vacutainer tubes with the gel separator and centrifuged within a few hours, as in good laboratory practice, the AMH results remained unaffected after many days at room temperature, even with robust handling.

The clinical deployment of AMH within assisted reproduction clinics is based upon research in specialized centres, mostly with on-site blood sample preparation without the use of postal/courier services. The distribution of AMH within the normal population shows a log-type distribution (Nelson et al., 2011) and clinical categorization of patients within clinics reflects this, as distinguishing values for poor-responder patients are in the region of 5–10 pmol/l, while values defining high-responder patients are in excess of 20 pmol/l (Nelson et al., 2009). Correspondingly, the evidence indicates that minor variations of AMH due to storage issues will have little impact on the clinical categorization. However, the worst-case evidence explored here, with a 31% increase in the measured value at 3.5 days, would result in a misleadingly high AMH value with potentially important clinical consequences.

These results have practical implications for centres using centralized AMH assay services and should be taken into consideration. Effectively, short-term storage (up to 24 h) is acceptable, but physical separation of the serum from the blood cells within a few hours of venepuncture is the ideal circumstance. The sample can then be couriered without the need for refrigeration and assayed at a remote site. This is best effected using gel separator tubes with centrifugation.

References


Declaration: RF, DL and MG are directors of a service providing AMH assays for clinics. CF and CB are employed by GCRM Labs, which provides that service.

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<table>
<thead>
<tr>
<th>Control AMH (pmol/l)</th>
<th>Experimental (T7) AMH (pmol/l)</th>
<th>Increment (%)</th>
<th>P (paired t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control sample = concentration obtained from separated serum centrifuged within 3 h of collection and assayed within 24 h of venepuncture. Experimental sample = prolonged storage (5 days at 20°C and 2 days at 4°C) followed the centrifugation step.</td>
<td>16.4</td>
<td>1</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 2: Effect of separating serum from blood cells on AMH concentration.