4-chloro-m-cresol test – a possible supplementary test for diagnosis of malignant hyperthermia susceptibility

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Background: In vitro contracture test (IVCT) for diagnosis of MH in our laboratory has a sensitivity of 100% and a specificity of 93%. The results are equivocal in 10–15%, and supplementary tests may thus be required. We have tested the hypothesis that 4-chloro-m-cresol (4-cmc) may be useful for a supplementary test.

Methods: Muscle from 41 consecutive patients from 7 families undergoing diagnostic muscle biopsy with IVCT was exposed in vitro to increasing concentrations of 4-cmc (25, 50, 75, 100, 150, and 200 pmol l⁻¹), and the force development recorded. Diagnosis of MH susceptibility was made with standard halothane and caffeine tests and included as results MHS (MH susceptible), MHN (MH negative), and MHE (equivocal result).

Results: At all concentrations of 4-cmc, the increase in baseline force was significantly greater in the MHS group compared to the MHN group (P<0.05). Muscle from 15 MH-susceptible (MHS) patients responded to 4-cmc with increasing force at a threshold concentration of 75 pmol l⁻¹ or less, whereas muscle from 23 MH-non-susceptible (MHN) patients had thresholds of 100 pmol l⁻¹ or more. The accuracy of the chlorocresol test was thus 100% (95% confidence limits 90.75–100%) at a threshold of 75 pmol l⁻¹. Amplitude of contractures at 2 mmol l⁻¹ caffeine was not different from contractures at 75 pmol l⁻¹ of 4-cmc in either the MHS or the MHN group (P>0.05). In vitro contractures of chlorocresol from clinical use of insulin and somatropin are estimated to be 20 times less than the threshold concentration and thus these drugs seem safe in MH patients.

Conclusion: Chloro-m-cresol may be a suitable aid to clarify puzzling results of standard testing of MH susceptibility.

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MalNignant hyperthermia is a pharmacogenetic disease of skeletal muscle. Because anaesthesia of susceptible individuals may lead to a fatal outcome if inhalational anaesthetics or suxamethonium are used it is desirable to establish an exact diagnosis in anybody suspected to be MH susceptible. We have for many years used the European MH Group (EMHG) protocol for this purpose (1, 2). The in vitro contracture test is based on cumulative dose-response curves to halothane and caffeine. The sensitivity of this test in our laboratory is 100% and the specificity 93% (3), which is acceptable for any biological test. However, this means that the risk of a false positive diagnosis is at least 7%. In 10–15% of those tested the result is equivocal (MHE). For reasons of safety, these patients most often are regarded clinically as MH susceptible.

One locus for inheritance of MH is the ryanodine receptor gene on chromosome 19, and until now, 8 mutations in this gene have been published (4–10). These mutations seem to account for MH in about 20% of human MH families, with some geographical variations in frequency. In addition, several loci on other chromosomes have been tentatively related to MH, although no specific mutations have yet been determined. Correct phenotyping is crucial for genetic research as well as for understanding the pathophysiological changes any mutation may induce.

With a 7% risk of a false positive diagnosis, the standard halothane and caffeine tests must be supplemented by other tests to establish a correct phenotype, or other ways to estimate risk must be introduced, as for example Bayesian statistical evaluation of results of standard tests. A ryanodine contracture test has been suggested, but not universally accepted (11, 12). In our laboratory we have observed some overlap in results of the ryanodine test between the groups of MHS and MHN individuals (unpublished results). Recently, it was suggested that 4-chloro-m-cresol (4-cmc) may be a more useful compound for a contracture test (13). Chlorocresol is the solvent compound
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responsible for the contractures originally attributed to suxamethonium (14). It releases calcium from skeletal muscle calcium release channels like caffeine does, but perhaps through binding sites different from those of caffeine (13). It may therefore be a preferred supplementary test for MH. In the present investigation we have tested the hypothesis that 4-chloro-m-cresol may be used to distinguish between MH-susceptible and MH-negative individuals.

Material and methods

Forty-one consecutive patients from 7 families undergoing diagnostic muscle biopsy with IVCT at our centre participated in the study after informed consent was obtained. The reason for investigation was fulminant MH in a family member (n=37), abortive MH in a family member (n=1), and clinically suspected MH during anaesthesia in the patient himself (n=3). The study protocol adheres to the standards described in the declaration of Helsinki and was approved by the Ethics Committee of Copenhagen County.

Diagnosis of MH susceptibility was made with standard duplicate static cumulative halothane and caffeine tests according to the protocol of the EMHG (1, 2). In addition, one halothane 3% bolus test was performed as described by the North American MH group (15). If any muscle was left, a ryanodine test using 1 μmol l⁻¹ was also performed (11).

One extra piece of muscle was excised from each patient specifically for this investigation. This piece of muscle was exposed in a separate tissue bath to increasing concentrations of 4-chloro-m-cresol. A stock solution of 4-chloro-m-cresol (Fluka Chemie, Neu-Ulm, Germany) of 25 mmol l⁻¹ was prepared in distilled water. Test solutions were prepared from the stock solution by diluting appropriate amounts in Krebs solution. The final concentrations of 4-cmc used were 25, 50, 75, 100, 150, and 200 μmol l⁻¹.

In the tissue bath, the muscle was electrically stimulated with a supramaximal current at 0.2 Hz. The muscle was stretched to optimal length and left to stabilise at this length for 10–20 min. The baseline force was continuously recorded. When baseline force was stable, 4-chloro-m-cresol was introduced into the tissue bath.

In preliminary experiments with 4-cmc, we observed some variability in the muscle response to the compound. Most often, a rapid increase in baseline force was followed by a decline which varied in shape. Maximum contracture was reached within 3–5 min. This type of response was similar to the caffeine response. In a few samples, however, force increased slowly, sometimes with a linear curve shape, for up to 20 min. Based on our preliminary experiments, we decided to expose the muscle to each concentration of 4-cmc for 6 min, not trying to add the next concentration at the height of the contracture response, as is usually done in the caffeine test, and not trying to define a stable increase in force. With exposure for 6 min, we were unlikely to miss any contractures. Thus, in all tests performed in this study, after exposure of the muscle for 6 min to any concentration of 4-cmc, the next concentration was added, independent of the response to the previous concentration.

All tests were performed at 37°C. A threshold concentration of chlorocresol was defined as the concentration at which baseline force increased 0.2 g or more.

In addition to IVCT, blood was drawn for DNA analysis. The patients were tested for the presence of 6 of the 8 published mutations: Arg163Cys (6), Gly341Arg (8), Ile403Met (6), Arg614Cys (4), Gly2433Arg (9), and Arg2434His (7).

To estimate if chlorocresol plasma concentrations could reach dangerous levels during treatment of MHS patients with drugs preserved with chlorocresol, the Danish Pharmacopoeia was searched for drugs preserved with chlorocresol and the content of chlorocresol noted. Estimated maximal plasma concentrations of chlorocresol were calculated, assuming instantaneous absorption into a plasma volume of 3 l for a dose in the upper range of those clinically used.

Data are reported as mean values with standard deviations. For binomially distributed data probability with 95% confidence limits are given. Individual dose-response curves were drawn from the non-linear regression lines. Data were compared using two-way analysis of variance for repeated measurements, followed by multiple comparisons using the Student-Newman-Keuls method. In addition, the Mann-Whitney test, and the Wilcoxon matched-pairs signed-ranks test were used, as appropriate. All tests were made using the SigmaStat software (16). P<0.05 was considered significant.

Results

In all individuals participating in this investigation, duplicate, cumulative halothane and caffeine tests, a single 3% halothane bolus test, and a single test with 4-chloro-m-cresol were performed. In 19 patients, a ryanodine test was also performed. The standard IVCT showed 15 individuals to be MHS, 23 MHN, and 3 MHE (with 2 patients having abnormal halothane test results (MHEh) and 1 patient abnormal caf-
Chlorocresol test for MH

Table 1

Results of IVCT in 38 patients investigated for MH (15 MHS and 23 MHN). Results are mean values with standard deviations in brackets.

<table>
<thead>
<tr>
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<th>Contracture at 2% halothane, g</th>
<th>Contracture at 2 mmol l⁻¹ caffeine, g</th>
<th>Contracture at 3% halothane bolus, g</th>
<th>Time to 1 g contracture at ryanodine 1 µmol l⁻¹, min</th>
<th>Contracture at 75 µmol l⁻¹ chlorocresol, g</th>
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<tbody>
<tr>
<td>MHS</td>
<td>2.17 (1.16)</td>
<td>1.07 (0.51)</td>
<td>3.99 (1.59)</td>
<td>6.96 (2.68)</td>
<td>1.11 (0.97)</td>
</tr>
<tr>
<td>MHN</td>
<td>-0.23 (0.16)</td>
<td>-0.18 (0.15)</td>
<td>-0.01 (0.31)</td>
<td>19.25 (—)</td>
<td>-0.28 (0.11)</td>
</tr>
</tbody>
</table>

*indicates significant difference between MHS and MHN. Ryanodine test was only performed in 2 MHN patients, and thus no statistical comparison could be made.

feine test results (MHEc)). None of the mutations tested for were found in any of the patients. Details of the IVCT results for MHS and MHN patients are shown in Table 1.

In muscle from 31 patients, contractures were observed during the 4-cmc test, whereas baseline force did not increase in muscle from 10 MHN patients, even at a 4-cmc concentration of 200 µmol l⁻¹. Maximum contractures were significantly greater at all concentrations of 4-cmc in the MHS group compared to the MHN group (P<0.05). The 4-cmc dose-response curves are shown in Fig. 1.

Muscle from all 15 MH-susceptible (MHS) patients responded to 4-chloro-m-cresol with increasing force at a threshold concentration of 75 µmol l⁻¹ or less, whereas muscle from all 23 MH-non-susceptible (MHN) patients had thresholds of 100 µmol l⁻¹ or more. Thus, there was no overlap between MHS and MHN patients regarding the chlorocresol threshold concentration. The threshold concentration was 25 µmol l⁻¹ for 1 MHS patient, 50 µmol l⁻¹ for 10 MHS patients and 75 µmol l⁻¹ for 4 MHS patients. Although the contractures at all concentrations of chlorocresol were significantly greater in the MHS group, an overlap in contracture size was seen between individual MHS and MHN patients at chlorocresol concentrations above 100 µmol l⁻¹. The contractures elicited at the critical concentrations of caffeine and 4-
Contracture (g)

![Graph showing contracture size](image)

**Fig. 2.** Contracture size of muscle from 15 MHS and 23 MHN patients at exposure to caffeine 2 mmol l⁻¹ and 4-chloro-m-cresol 75 μmol l⁻¹. For each group of patients, the responses to caffeine and chlorocresol were not significantly different (*P* > 0.05, Wilcoxon matched-pairs signed-ranks test).

chloro-m-cresol, 2 mmol l⁻¹ and 75 μmol l⁻¹, respectively, were similar within each diagnostic group (*P* > 0.05) as shown in Fig. 2.

Three patients (7.3%) had equivocal results of the IVCT. One of these (MHEh) had a chlorocresol threshold of 50 μmol l⁻¹, thus indicating a possible status of MH susceptibility, whereas muscle from the other two had thresholds of 100 and 150 μmol l⁻¹, respectively, which supports the view that they are not MH susceptible. Individual results from all types of tests performed in these 3 patients are shown in Table 2.

The results of the 3% halothane bolus test and the ryanodine test support the view that one of these individuals is truly MH susceptible whereas the other two are MH negative.

Applying the diagnostic criterion of + / − contracture at 4-chloro-m-cresol 75 μmol l⁻¹ or less in the patients with a definite diagnosis according to our present diagnostic criteria (MHS and MHN patients) gives an accuracy of the chlorocresol test of 100% with 95% confidence limits of 90.75–100%.

We found that chlorocresol is used for preservation of insulin and somatropin in Denmark at a concentration of 0.1%. With a molecular weight of 142.59 of the chlorocresol compound used in these drugs (personal communication, the Hospital Pharmacy of Copenhagen County), a dose of 1 ml (with a content of 1 mg of chlorocresol) of insulin or somatropin could thus theoretically result in a plasma concentration of chlorocresol of about 2.3 μmol l⁻¹. This is 10–20 times less than the concentration which in vitro elicited muscle contractures in MHS patients (for most of the patients, 50 μmol l⁻¹).

**Discussion**

A precise diagnosis of susceptibility to malignant hyperthermia (MH) is highly desirable, both for patient safety and for solving the genetic problems of MH. IVCT following the protocol of the European MH Group in our laboratory has a sensitivity of 100% and a specificity of 93% (3). It cannot be expected that any test will be both 100% sensitive and 100% specific, and the IVCT as presently performed in Europe seems satisfactory. The results of IVCT for diagnosis of MH susceptibility depend on the methods used for the test (3). Even with a great degree of standardisation, as has been obtained by the European MH Group, it appears that there are differences among individual laboratories in results, the causes of which are not known. Thus the frequency of the MHE result varies (internal review of the EMHG). Part of this variation may be due to genetic differences, since the specific mutations associated with MH are observed with dif-

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<tr>
<td>No. 1</td>
<td>0.3</td>
<td>0.05</td>
<td>0.65</td>
<td>16.0</td>
<td>0.1</td>
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<tr>
<td>No. 2</td>
<td>0.5</td>
<td>0.1</td>
<td>2.25</td>
<td>9.5</td>
<td>0.3</td>
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<tr>
<td>No. 3</td>
<td>0</td>
<td>0.55</td>
<td>0.95</td>
<td>14.7</td>
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ferent frequencies in individual European countries. Also, the MHE phenotype is found significantly more often in probands having had signs of MH during anaesthesia than in normal controls (17). Therefore, other genes than those directly causing MH may perhaps modify the functional status of the calcium release channel (18), and may also, perhaps, modify an individual’s response to anaesthetics and thus the response to tests for MH. However, no such genes are known at present.

In order to characterise the various phenotypes of MH, and to discover the expected false positive test results, it is important to characterise individuals by more than one test. On the other hand, this will increase the risk of obtaining at least one abnormal test result. Therefore, it seems wise to rely on the standard halothane and caffeine tests for pure diagnostic purposes, and then add other tests on an optional basis for research.

In the present investigation we found 4-chloro-m-cresol to be useful for differentiation between MHS and MHN individuals, with no overlap in results between the groups, when the 4-cmc threshold concentration was used as the critical parameter and a value of 100 μmol L⁻¹ or more was considered normal. Herrmann-Frank et al. originally described the chlorocresol contracture test in a larger study of the effects of chlorocresol on the muscle calcium release channel (13). She performed the test in 20 specimens but did not specify the number of individuals from whom the muscle samples were taken. She found that muscle from MHN patients developed contracture at ≥75 μmol L⁻¹, whereas MHS specimens developed contracture already at 25 μmol L⁻¹. Our results are partly similar to hers in that we could separate MHS and MHN individuals by the chlorocresol test, partly differing in that the concentrations eliciting contractures were higher in our laboratory than in hers. The dose-response curve for MHS, however, seems to be similar, except that we obtained larger contractures at 200 μmol L⁻¹. In another study including 11 patients investigated for MH susceptibility (19), the 4-chloro-m-cresol dose-response curve was virtually identical to that obtained by us. The finding of different threshold concentrations, however, makes it absolutely necessary to establish individual reference values within each laboratory for this test. It is a potential weakness of our study that we used only one specimen from each patient for the test with 4-cmc. However, we found it of more benefit to use different agents and methods of adding the drugs rather than repeating tests and hence continued to make one test with halothane 3% given as a bolus dose and, if muscle was left, one test with ryanodine. It is clinically impractical to add drugs to the test armamentarium and require duplicate or triplicate analyses for all drugs. Thus, the choice stands between single tests with several different drugs or several repeat tests with one drug in addition to the standard tests with halothane and caffeine. We prefer the first option.

In our laboratory, we have observed overlap between the groups of MHS and MHN, both when using the 3% halothane bolus test (3) and the ryanodine test (unpublished results). This was not the case with the 4-cmc test, which therefore seems promising as an extra test for MH. Our three MHE results could be differentiated into two normal and one abnormal result with the chlorocresol test. We have not yet found any specific mutations in the patients participating in this study, so we cannot relate the presence or absence of a mutation to the results of the IVCT.

It is not known precisely how 4-chloro-m-cresol releases calcium from the sarcoplasmic reticulum. Our finding of similar contractures at 2 mmol L⁻¹ caffeine and 75 μmol L⁻¹ 4-chloro-m-cresol may indicate that the action is similar to that of caffeine, and in this case a test with 4-chloro-m-cresol may not offer any advantages compared to the caffeine test. Another explanation for the similarity of results could be that both drugs act at steps “higher up” in the cascade of events leading to release of calcium (18). The MHE patient with abnormal caffeine results, however, had a normal 4-cmc threshold. This may indicate that the action of caffeine and 4-cmc is not identical. However, it may also be due to the variation in individual test results which is sometimes observed.

The finding of contracture responses in MHS muscle exposed to micromolar concentrations of 4-chloro-m-cresol might theoretically have a clinical impact because chlorocresols are used as preservative agents in some drugs. In Denmark, chlorocresols are found only in insulins and somatropins. However, our estimate of maximal plasma concentrations of chlorocresols following normal use of insulin and somatropin indicates that drugs preserved with chlorocresols are safe even in MHS patients because concentrations eliciting contractures in vitro in MHS muscle are approximately 20 times higher than the calculated maximal plasma concentration of chlorocresol.

In conclusion, we found an in vitro test with increasing doses of 4-chloro-m-cresol able to accurately distinguish between MHS and MHN patients. This test therefore may be a useful additional test for more detailed phenotyping of patients with equivocal or possibly false positive results of the standard tests for MH susceptibility.
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References


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