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Reproducibility of *in vitro* contracture test results in patients tested for malignant hyperthermia susceptibility

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Background: The *in vitro* contracture test (IVCT) is the golden standard to diagnose malignant hyperthermia susceptibility (MHS). A high reproducibility is important for a high validity of a test.
Methods: We have therefore analyzed IVCT in 838 patients, investigated in two laboratories. Each halothane and caffeine test was performed in two muscle strips. The test results were analyzed with respect to reproducibility of abnormal outcomes within pairs of tested muscle strips and size of contractures, thresholds and quality criteria. The patients were tested according to the European Malignant Hyperthermia Group protocol (EMHG). To fulfill quality criteria in the EMHG protocol the twitch height should be 10 mN (1 g) or more. For the caffeine test a minimum contracture of 50 mN (5 g) or more at 32 mmol l^{-1} caffeine could be used as an alternative quality criterion
Results: There was better reproducibility with larger contractures. The correlation between size of contractures and fraction of muscle strips with abnormal contractures was 0.77 or larger. Contractures < 5 mN (0.5 g) were reproducible in less than half of the tests. There was no difference in reproducibility or size of contractures between tests fulfillling all quality criteria and those not fulfillling these criteria.
Conclusions: IVCT responses close to cut off limits, i.e. <5 mN (0.5 g) in the EMHG protocol, are less reproducible and must scientifically be considered as less reliable. The clinical cut off limits must remain unchanged for reasons of clinical safety. The outcome of quality measurements does not influence the test results.

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Key words: caffeine halothane contracture test; diagnosis; *in vitro* contracture test; malignant hyperthermia; muscle; reproducibility.

Malignant hyperthermia susceptibility (MHS) is a pharmacogenetic disorder of intracellular calcium homeostasis. In susceptible individuals halogenated inhalational anesthetics and/or suxamethonium may trigger a malignant hyperthermia (MH) reaction, which is characterized by a potentially life-threatening hypermetabolism. MHS is diagnosed with the *in vitro* contracture test (IVCT) of biopsied skeletal muscle. If a proband is diagnosed MHS in IVCT and genetic analyzes reveals a known MHS mutation then mutation analysis can be used for making the MHS diagnosis in relatives. However mutation analysis can never be used for excluding MHS (1).

In the IVCT viable muscle strips are exposed to halothane and caffeine, respectively. The European Malignant Hyperthermia Group (EMHG) has a common protocol for IVCT (2, 3). Sensitivity and specificity of the IVCT have been established with test results from patients who survived a fulminant MH reaction and control individuals. There is a wide variation in the size of contractures and the fraction of muscle strips with abnormal contractures obtained in patients with positive IVCT. The cut-off limits have deliberately been chosen to obtain maximum sensitivity for the reason of clinical safety, to the cost of a lower specificity. However, in scientific investigations it is often important with a specificity close to 100%. We hypothesize that a high reproducibility of the IVCT may be linked to a high specificity and therefore we have studied the reproducibility of IVCT and the influence of quality criteria on test results in 838 patients from two laboratories.

Material and methods

We have retrospectively investigated the outcome of halothane static and caffeine tests in IVCT in 838 consecutively tested patients in two laboratories (1676 pairs of muscle strips). A flow sheet for screening of data is presented in Fig. 1. Forty-seven pairs of muscle strips were excluded from the study due to rupture or technical problems. Eleven hundred-five pairs of

muscle strips from 600 patients are not included since they revealed normal results (contracture <2 mN) in all musclestrips. All probands were investigated for other neuromuscular diseases, and relatives were so if they exhibited any symptoms or signs of such a disease. In this series no patients were found to suffer from other neuromuscular diseases than MH.

The IVCT was performed according to the protocol of the EMHG (2, 3). All tests were performed simultaneously in duplicate. Halothane and caffeine tests were performed in separate baths. All patients in each laboratory were tested with the same equipment and by the same staff. Both laboratories have similar equipment with bath volumes of ~5 ml and continous flow of Krebs/caffeine solution. All tests were performed within five hours. Halothane and caffeine concentrations were measured according to the EMHG protocol. Laboratory A also performs an additional halothane dynamic test according to the EMHG protocol. Diagnoses in laboratory A are based on the outcome from both halothane tests.

To fulfil quality criteria in the EMHG protocol the twitch height should be 10 mN (1 g) or more before exposure to halothane or caffeine. For the caffeine test a minimum contracture of 50 mN (5 g) or more at 32 mmol l^{-1} caffeine could be used as an alternative quality criterion The contracture, threshold and twitch height for each muscle bundle was determined. An abnormal contracture is a sustained increase in muscle force of at least 2 mN (0.2 g) at 0.44 mmol l^{-1} (2%) halothane or 2 mmol l^{-1} caffeine or less. The threshold concentration is the lowest concentration of halothane or caffeine resulting in a contracture of 2 mN (0.2 g) or more (2, 3). Viability was measured by the twitch height. Twitches were elicited with 1 ms supramaximal stimulation at a frequency of 0.2 Hz. The twitch height measured, was the height of the twitches before exposure to halothane or caffeine. One hundred and sixty-eight patients were probands who suffered a fulminant or suspected MH reaction, the rest were relatives to a proband with a positive test result. The patients were consecutively tested during a period of six years.

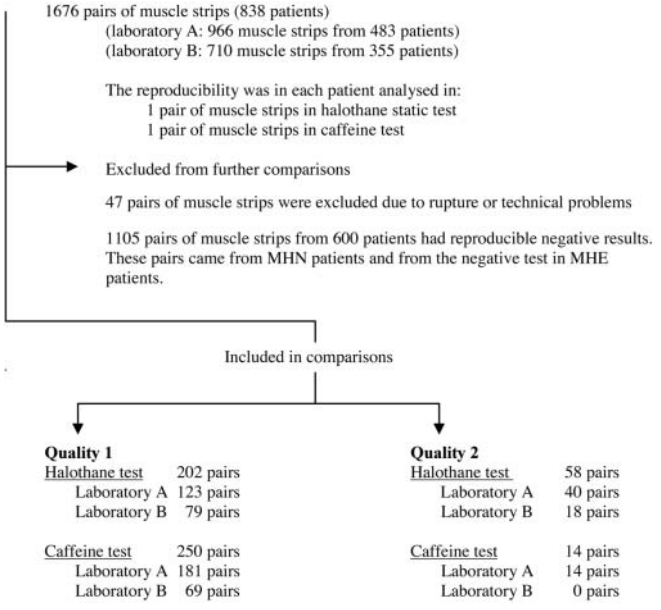

Fig. 1. The flowsheet indicates how consecutive IVCT test results were entered into the study. Reproducibility was analysed within pairs of muscle strips in each test. A positive test was considered reproducible if both muscle strips developed a contracture. MHN results are by definition reproducible. See text for further description.

Malignant Hyperthermia Susceptible (MHS) is diagnosed when there is a contracture of 2 mN (0.2 g) or more at 0.44 mmol l^{-1} halothane or less and a caffeine contracture of 2 mN (0.2 g) or more at 2 mmol l^{-1} caffeine or less; MHN (Malignant Hyperthermia Negative) is diagnosed when there is no contracture seen at 0.44 mmol l^{-1} halothane or 2 mmol l^{-1} caffeine or less; MHE (Malignant Hyperthermia equivocal) is the diagnosis in all other cases. A reaction to halothane only is labeled MHEh and to caffeine only MHEc.

Reproducibility in each pair of muscle strips was determined binominally, i.e. the test was considered reproducible if there was an abnormal contracture in both muscle strips tested. The contractures used for diagnostic purpose were the highest contracture obtained in the two muscle strips tested in a test. When further analysing the reproducibility, the sizes of contractures were divided into three groups, based on the results obtained among controls and when comparing reproducibility between laboratories and protocols and within monozygote twins (4–6). Group 1 consists of those with contractures within the range seen among our control individuals (>=2, <5 mN), group 2 (>=5, <10 mN) and group 3 reacting with at least twice the size of the contractures seen among our control individuals (>=10 mN). The reproducibility of the thresholds within pairs of muscle strips was evaluated. Quality was analyzed in each muscle strip, and the tests were divided into two groups; Quality 1 where both muscle strips fulfilled all quality criteria and Quality 2 where one or both muscle strips had a twitch height below 10 mN or a contracture to 32 mM caffeine below 50 mN.

For establishment of sensitivity and specificity, in the two laboratories, 19 patients, who suffered a fulminant MH reaction with MH rank 6 according to Larach (7), have been investigated following the protocol. All fulminant patients had a positive test result

and in 18 of these patients all contractures were reproducible irrespective of twitch height. The median contracture in the halothane test was 19 mN (range 2–8.9 mN) and in the caffeine test 11 mN (range 3–60 mN). The pooled material of control individuals tested according to the protocol in the two laboratories consists of 60 individuals, out of whom 3 were MHEh and 2 MHEc with contractures between 2 and 4.5 mN (8) the remaining were MHN. The results were reproducible in only one pair of muscle strips, while in the remaining four patients only a single muscle strip reacted.

Table 1

Size of contractures in different diagnostic groups in the two laboratories

	Size of contractures (mN) Median (range)	
Diagnoses/type of test	Laboratory A	Laboratory B
No. of patients	n = 483	n = 355
MHS	n = 163	n = 74
Caffeine	7 (2–98	8 (2–60)
Halothane	11 (2–76)***	15 (2–86)***
MHEh	n = 52	n = 33
Halothane	3 (2–9)	3 (2–12)
MHEc	n = 35	n = 6
Caffeine	4 (2–48)	3 (3–7)
MHN	n = 233	n = 242

*** In MHS patients statistically significantly larger contractures in halothane versus caffeine tests within each laboratory, $P < 0.001$.
MHS denotes a reaction in both halothane and caffeine test; MHEh denotes a reaction in only halothane test; MHEc denotes a reaction in only caffeine test; MHN denotes a negative test.

Table 2

Size of contractures in the two quality groups

	Size of contractures (mN)	
	Quality 1 median (range)	Quality 2 median (range)
Laboratory A		
Halothane	10 (2–76)	9 (2–31)
	n = 123	n = 40
Caffeine	6 (2–98)	5 (2–48)
	n = 181	n = 14
Laboratory B		
Halothane	10 (2–86)	6 (2–43)
	n = 79	n = 18
Caffeine	7 (2–60)	
	n = 69	n = 0

Quality 1: all muscle strips fulfilled required quality criteria. Twitch height ≥10 mN and/or in caffeine test a contracture to 32 mM larger than 50 mN; Quality 2: all muscle strips did not fulfil required quality criteria.
No significant difference in size of contracture between the two quality groups.

Statistics

Descriptive data are presented as median and range or 10th, 90th percentiles. Comparisons between patients were performed with Mann–Whitney *U*-test and within patients with Wilcoxon signed ranked sum test. Proportions were compared with Fishers' exact test or chi-squared test when applicable. Correlations were calculated with Spearman rank correlation test. $P < 0.05$ was considered significant.

Statistical tests were performed using GraphPad Instat version 3.00 and Stat mate, Graphpad Software, San Diego, CA (http://www.graphpad.com).

Results

Out of the 838 patients, 483 came from laboratory A and 355 from laboratory B. The male/female ratio were 0.95/1 and 0.93/1, respectively. The median age was 35 and 33 years, respectively. The diagnostic outcomes and contracture results are presented in Table 1.

The reproducibility of the caffeine and halothane tests are presented in Fig. 2. The results are for each test and laboratory divided into groups according to magnitude of contractures and quality of the muscle

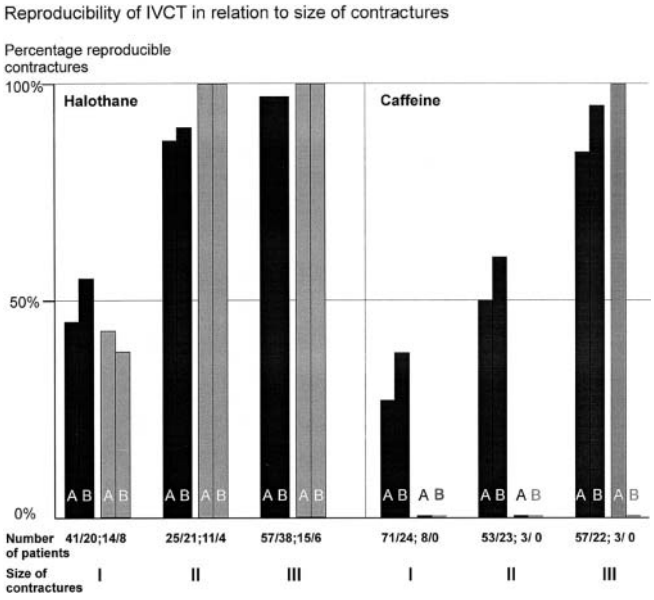

Fig. 2. Reproducibility of contractures in halothane and caffeine tests in two laboratories, A and B. A test result was considered reproducible if both muscle strips developed a contracture. Contractures are divided into three groups depending on size of the largest contracture in a pair of muscle strips with positive result. Reproducibility is significantly larger with increasing contracture size ($P < 0.01$) in both tests and laboratories. Both muscle strips fulfilled all quality criteria. Muscle strips did not fulfill all quality criteria. Size of contractures: I ≥2 mN, <5 mN; II ≥5 mN, <10 mN; III ≥10 mN. A, results from laboratory A; B, results from laboratory B.

strips. There was a higher reproducibility with increasing size of contractures ($P < 0.01$) in both tests and laboratories (Fig. 2). Reproducible contractures were significantly larger than not reproducible contractures, data presented in Fig. 3. However, there was no significant difference in reproducibility or size of contractures in pairs of muscle strips fulfilling quality criteria and those, which did not fulfil quality criteria (data presented in Table 2).

There was a uniform correlation >0.76 between the size of contracture in a test and the fraction of muscle strips with abnormal contractures. The correlation between contracture size and threshold value was uniform negative and below 0.74. The correlation between twitch height and contracture size was lower than 0.3 and varying between tests and laboratories. Data presented in Fig. 4.

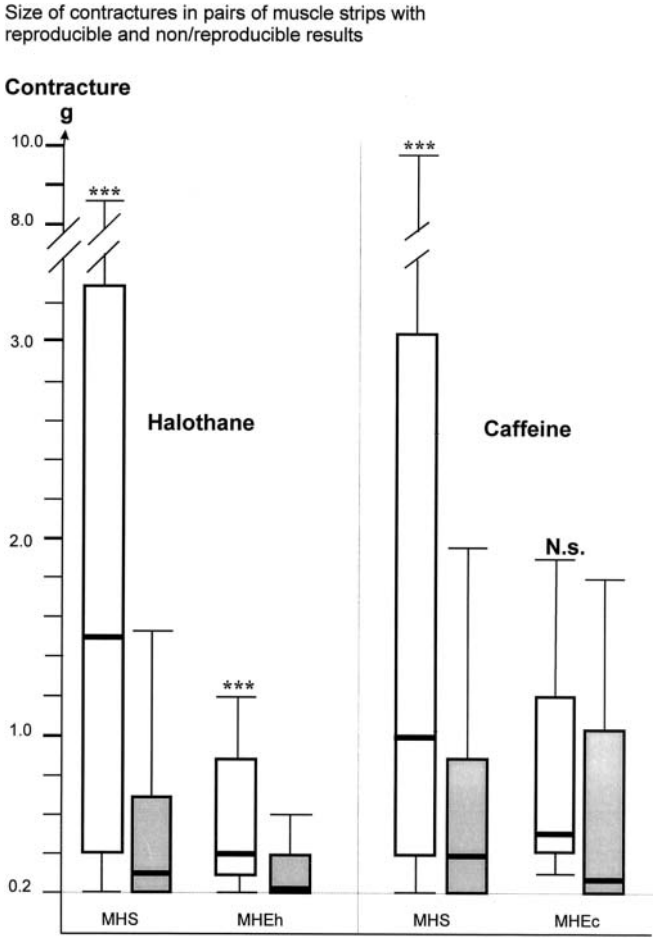

*Fig. 3. Comparison of size of contracture in muscle pairs where both muscle strips developed a contracture, versus pairs where only one muscle strip developed a contracture. Maximum contracture in each muscle pair is presented. Data presented as median and 10th/90th percentile and range. Size of contracture in reproducible tests; Size of contracture in non-reproducible tests. ***P < 0.001 in comparison between reproducible and non-reproducible results.*

Halothane contractures were significantly larger than caffeine contractures in both laboratories ($P < 0.01$). Halothane contractures were significantly more reproducible than caffeine contractures in laboratory A ($P < 0.01$). There was no such statistically significant difference in laboratory B, however, $P = 0.058$. There was no significant difference in reproducibility or size of contractures between males and females or patients who received regional or general anaesthesia (data not presented). Five patients with a fulminant reaction were tested in this material and they reacted with reproducible contractures, between 3 and 86 mN, irrespective of twitch height, and response to 32 mmol l^{-1} caffeine.

In tests with abnormal contractures the thresholds were identical in laboratory A in 59% of the halothane tests and 26% of the caffeine tests. The corresponding values in laboratory B were 61% and 34%. There was no significant difference in reproducibility in threshold values between laboratories ($P > 0.05$).

There was no difference in size of contractures or reproducibility between the static and dynamic halothane test in laboratory A ($P > 0.05$). In the MHEh group from laboratory A ($n = 53$) 60% reacted only in the dynamic test.

In summary:

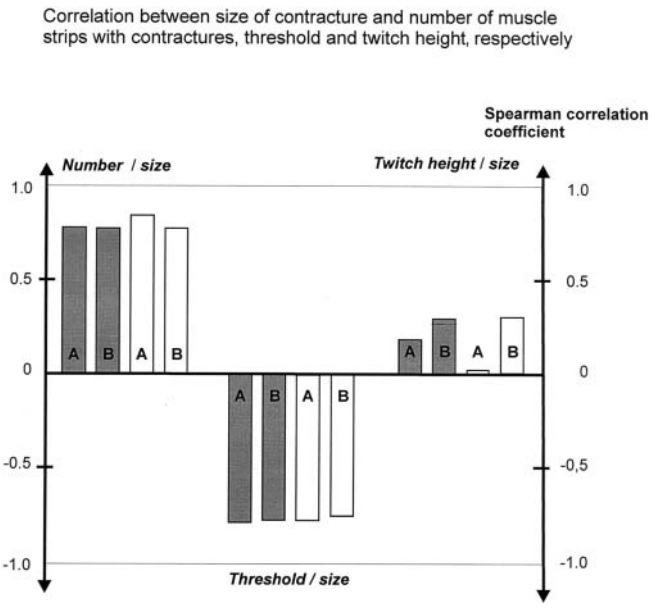

Fig. 4. Number denotes the fraction of muscle strips with abnormal contracture in an IVCT; Threshold denotes the concentration at which a contracture develops in a test; Twitch height denotes the size of twitches elicited by electrical stimulation; Correlated to: Size denotes the maximum size of contracture in halothane and caffeine test, respectively. Halothane test; caffeine test. A, results from laboratory A; B, results from laboratory B.

1. there was increasing reproducibility with increasing size of contracture;
2. the reproducibility of the results was the same in pairs of muscle strips fulfilling all quality criteria and those not fulfilling all criteria;
3. halothane contractures were more reproducible than caffeine contractures.

Discussion

Despite extensive studies, the IVCT remains the only test for diagnosing MHS in probands. For the reason of clinical safety, the threshold for abnormal reactions was deliberately chosen to obtain a high sensitivity at the price of a somewhat lower specificity, in both the EMHG and the NAMHG protocols. In the IVCT we found a strong and uniform correlation between size of contractures and fraction of muscle strips with abnormal contractures. A high reproducibility is fundamental for a high validity of a diagnostic test. We have therefore analysed the reproducibility within duplicate tests, as well as the reproducibility of tests in patients investigated simultaneously in two laboratories (4). Our findings suggest that the fraction of muscle strips with abnormal contractures and the size of the contractures should be taken into account when evaluating the validity of IVCT for scientific studies.

In a Scandinavian control material the sensitivity of the IVCT was 100% and the specificity 92% (8). In the combined European material of patients suffering previous fulminant MH and controls the sensitivity was 99% and the specificity 93.6% (3). In the same study the specificity was increased to 98% if MHE results were considered unknown. Few patients who suffered a fulminant reaction exhibit contractures below 5 mN (3). In our material there is an overlap of 2–4.5 mN between a few of our control individuals, compared with the results from other types of patients, e.g. probands with abortive reactions and relatives of probands (9). When comparing the outcome of halothane tests performed according to the EMHG and NAMHG protocol in the same patients we found a group with diverging outcome between the two tests (5). This group has mainly halothane contracture sizes between 2 and 4.5 mN which is within the range seen in non-reproducible tests, within patients or between laboratories and among control individuals (4, 6, 8), i.e. <5 mN. This phenomenon can not be explained only by laboratory inconsistencies but may reflect a different genetic background or yet unknown environmental factors.

There was no difference in reproducibility or size of contractures between tests fulfilling quality criteria and those, which did not fulfil these criteria. The twitch heights could be influenced by both internal and external factors. Examples of internal factors are the degree of intact cell membranes and sufficient energy supply. Examples of external factors are moderation of the signal by transducer and printer. Other quality criteria have been suggested, e.g. measurement of membrane potentials (10). Iaizzo et al. found that IVCT results were independent of resting membrane potential (11). Adnet et al., on the other hand, found muscle strips less sensitive to caffeine with less resting membrane potential in patients with neuromuscular diseases (10). These contradictory results indicate that there is no proven way of measuring the quality of muscle strips used in IVCT.

Lack of reproducibility in biological measurements can be due to several factors (12): (i) Imprecision or experimental error. This is often a small source of variability in medical studies; (ii) Biological variability. In biological and clinical studies scatter is often due to biological variation; (iii) Blunders, mistakes and glitches.

Laboratory imprecision is one source of variability in the IVCT that is not negligible. There could be deviations from the protocol in concentrations of halothane or caffeine, temperature or pH, despite meticulous standardization and frequent measurements. The surgical technique, handling of muscle strips and performance of the IVCT can differ between individuals. However, this can not be the only explanation of variability in test results in this study. Few individuals have taken the biopsies and performed the IVCT in a standardized way, and we have not observed any deviations in concentrations of test drugs at our regular measurements of these.

Another explanation of the variability in the IVCT results could be different distribution of fiber types in the muscles and/or muscle strips. However, no correlation has been found between distribution of fiber types and size of contractures (13, 14). These results indicate that distribution of fiber types is not important for the outcome of IVCT.

For clinicians it is of utmost interest to exclude MH susceptibility in a patient with suspected MHS whenever possible, since the use of trigger agents is sometimes desirable. In scientific work, e.g. identification of MHS genes, it is important to include only true MHS individuals, since a correct phenotypic diagnosis is fundamental for obtaining a correct genotype. To further refine the diagnostic outcome, complementary tests such as the ryanodine test (15) and the 4-chloro-cresol (16) test have been used. However, few

patients suffering fulminant MH have been tested with these agents and so the possible benefit of these tests is not yet settled. So far these additional tests do not seem to discriminate MHS from MHN better than the standard IVCT. In already investigated kindreds suitable for genetic studies the only way to refine the diagnoses is to further assess the IVCT data.

In conclusion, there was no difference in reproducibility or size of contractures in tests fulfilling and those not fulfilling the required quality criteria. Halothane tests were more reproducible than caffeine tests. Contractures larger than 5 mN were reproducible in more than 87% of the halothane tests. The diagnoses in patients with non-reproducible contractures less than 5 mN should be regarded as unknown in scientific studies. However, due to patient safety the cut-off limit must remain unchanged for clinical diagnosis.

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