Modified iodine-paper technique for the standardized determination of sweat gland activation

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Gagnon D, Ganio MS, Lucas RAI, Pearson J, Crandall CG, Kenny GP. Modified iodine-paper technique for the standardized determination of sweat gland activation. J Appl Physiol 112: 1419–1425, 2012. First published February 9, 2012; doi:10.1152/japplphysiol.01508.2011.—Quantifying sweat gland activation provides important information when explaining differences in sweat rate between populations and physiological conditions. However, no standard technique has been proposed to measure sweat gland activation, while the reliability of sweat gland activation measurements is unknown. We examined the interrater and internal reliability of the modified-iodine paper technique, as well as compared computer-aided analysis to manual counts of sweat gland activation. Iodine-impregnated paper was pressed against the skin of 35 participants in whom sweating was elicited by exercise in the heat or infusion of methylcholine. The number of active glands was subsequently determined by computer-aided analysis. In total, 382 measurements were used to evaluate: 1) agreement between computer analysis and manual counts; 2) the interrater reliability of computer analysis between independent investigators; and 3) the internal reliability of sweat gland activation measurements between duplicate samples. The number of glands identified with computer analysis did not differ from manual counts (68 ± 29 vs. 72 ± 24 glands/cm2; P = 0.27). These measures were highly correlated (r = 0.77) with a mean bias ± limits of agreement of −4 ± 38 glands/cm2. When comparing computer analysis measures between investigators, values were highly correlated (r = 0.95, P < 0.001) and the mean bias ± limits of agreement was 4 ± 18 glands/cm2. Finally, duplicate measures of sweat gland activation were highly correlated (r = 0.88; P < 0.001) with a mean bias ± limits of agreement of 3 ± 29 glands/cm2. These results favor the use of the modified-iodine paper technique with computer-aided analysis as a standard technique to reliably evaluate the number of active sweat glands.

Sweat production from eccrine glands is arguably the most important effector mechanism involved in human temperature regulation during heat stress. It is therefore not surprising that normal sweat rate responses have been thoroughly characterized at rest and during exercise (25, 27, 30, 40), with many investigations examining how sweat production differs between populations (3, 20, 22, 33, 41), as well as with various physiological (23, 28, 38) and disease (24) states. Furthermore, recent work has established a valid method to assess the physiological control of sweating (9), as well as examine its normal biological variation (19).

Sweat is produced by 2–4 million eccrine sweat glands dispersed over the nonglabrous skin regions of the human body (34, 35). As such, total sweat production is determined by both the number of activated glands, as well as the output per individual gland (7, 25). Assessments of sweat gland activation have been used clinically to evaluate the extent of neurological damage caused by various disease states (13), as well as experimentally to evaluate sweat gland function in relation to exercise intensity (26), exercise training (8, 12), age (2, 14–17, 21, 39), obesity (5), and sex (5, 11, 18, 29). Importantly, the number of active sweat glands has also been used to determine whether differences in sweat rate between populations/conditions are mediated via central or peripheral mechanisms (36, 37).

To date, experimental assessments of sweat gland function have been achieved through a variety of techniques, including the starch-iodine technique (12, 15–18, 25, 26, 29, 39), the modified iodine-paper technique (5, 8, 11, 36, 37), and the macrophotographic technique (2, 21). Briefly, the starch-iodine technique consists of painting the skin surface with iodine and subsequently applying starch paper. The modified iodine-paper technique consists of applying iodine-impregnated paper onto the surface of unpainted skin. In both cases, the active sweat glands produce identifiable blue dots on the starch/iodine-impregnated paper. In contrast, the macrophotographic technique consists of painting the skin with Vaseline to promote beading of sweat while a series of pictures are taken from the measurement area. Common to all techniques is that the number of active sweat glands is subsequently counted manually, the count typically performed by the same experienced investigator.

Given the valuable information provided by measurements of sweat gland activation, it is surprising that no standard technique has been advocated to experimentally determine the number of active sweat glands. Considering that multiple laboratories utilize various techniques to achieve a common objective, the use of a standard technique would provide measurement consistency and allow direct comparisons between laboratories. Furthermore, interrater reliability of measuring the number of active sweat glands has not been examined. Since determining the number of active sweat glands within a collected sample is most often achieved through manual counts, it is unknown whether the number of active sweat glands determined by one investigator can reliably be interpreted by other external laboratories. With these issues in

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mind, we sought to evaluate the modified iodine-paper technique, in combination with a free, publicly available computer program (ImageJ), to count the number of active sweat glands. Specifically, we investigated 1) the agreement between computer-assisted and manual counts of active sweat glands; and 2) whether computer-assisted analysis is reliable between investigators from independent laboratories. Furthermore, we were interested in using the modified iodine-technique to determine the internal reliability of duplicate active sweat gland measurements. We hypothesized that the modified iodine-paper technique with computer-assisted analysis would provide a standardized and reliable means of determining the number of active sweat glands.

METHODS

Ethical Approval

The experimental protocol was approved by the University of Ottawa Health Sciences and Science Research Ethics Board, as well as the Institutional Review Boards of the University of Texas Southwestern Medical Center and Texas Health Presbyterian Hospital Dallas. Written informed consent was obtained from all volunteers before their participation in the study.

Subjects

The subjects consisted of 35 volunteers (13 females). All subjects were healthy, nonsmoking, and free of any known cardiovascular, metabolic, and respiratory diseases. The subjects had a mean ± SD age of 34 ± 11 yr, body height of 172 ± 11 cm, and body mass of 78.1 ± 16.9 kg.

Experimental Design

Study 1. Subjects were part of a larger study examining sex-related differences in temperature regulation. Upon arrival at the laboratory, the subject changed into shorts and sandals (as well as a sports bra for differences in temperature regulation. Upon arrival at the laboratory, the subject was transferred to an environmental chamber regulated to an ambient air temperature of 40°C and a relative humidity of 20%. A chest on the pectoralis major muscle, the upper trapezius muscle, and glands on the upper back, chest, and forearm was determined in duplicate at 30, 60, and 90 min of exercise using the technique described below. This experimental protocol was performed before and following 7 days of heat acclimation.

Measurement of Sweat Gland Activation

For both studies, the number of active sweat glands was determined using the modified iodine-paper technique originally proposed and validated by Randall (31, 32) and subsequently modified by Davis et al. (10). Four to five days before an experimental session, pieces of 100% cotton paper (32 lb; Southworth, Agawam, MA) were cut to a predetermined size (9 cm² in study 1; 2.83 cm² in study 2) and placed in a sealed container containing iodine in solid form (Sigma-Aldrich, St. Louis, MO). Each piece of cotton paper was supported to avoid direct contact with the iodine. After ~48 h, the pieces of paper became saturated with iodine (as indicated by their dark brown color) and were subsequently transferred to a sealed bag for use during the experimental protocol. To ensure a uniform application on the skin surface, double-sided tape was used to affix the cotton paper to a flat hard plastic surface. Before the application of the cotton paper the skin was blotted dry, following which the cotton paper was firmly pressed against the skin surface for a period of ~5 s. With the use of this technique, sweat excreted from the active sweat glands forms easily identifiable blue dots on the iodine-impregnated paper when it is placed in contact with the skin surface. After the paper was removed from the skin, it was immediately scanned at high resolution (600 dots/in.) using a commercially available scanner for subsequent analysis using the ImageJ image processing and analysis program (1).

Image processing and analysis. ImageJ is a public domain Java image processing and analysis program that can be downloaded for free (http://rsbweb.nih.gov/ij/index.html, last accessed November 20, 2011) on any operating system. ImageJ is particularly useful for determining the number of active sweat glands, as it can easily identify the number of individual particles from a scanned image. The reader is referred to the APPENDIX for step-by-step instructions on how to analyze sweat gland activation samples. It is important to note that the analysis requires the investigator to define a lower and upper size limit for the pixel area, which is the minimum/maximum size allowable for a dot to be considered in the count. In the current study, the investigators were allowed to choose the limits they deemed appropriate to ensure that all sweat glands were included within the count. Once the analysis performed, the software generates a count of the particles present in the image, which is the number of active glands for that sample (see Fig. 1). The number of glands is then divided by the surface area of the paper to give a value of active sweat glands per square centimeter.

Data Analyses

The following terms were used to describe comparisons made in the current study: 1) agreement: variability in sweat gland activation measurements between computer-assisted analysis and manual counts; 2) interrater reliability: the variability in sweat gland activation counts as determined by computer-assisted analysis between independent investigators; and 3) internal reliability: the variability of duplicate sweat gland activation measurements (determined using computer-assisted analysis).

A wide range of sweat gland activation measures were obtained by combining data from study 1 and study 2 and analyzed as one data set to answer the following questions: 1) is there good agreement between computer software and manual analyses when determining the number of active sweat glands; and 2) is computer-assisted analysis...
Comparative counts and then averaged together to provide an overall glands present. The coefficient of variation was calculated for each

question. The smallest change worth detecting with the current method was calculated using the formula: \( 1.96 \times \sqrt{2 \times SE} \), where \( SE \) stands for standard error of measurement, which itself was calculated using the formula: \( SD \times \sqrt{1-ICC} \) where \( SD \) stands for standard deviation and ICC for intra-class correlation coefficient of duplicate measurements. SigmaPlot 12.0 and Microsoft Excel 2010 were used for all analyses. Alpha was set at 0.05 for all statistical tests. Data are reported as means ± SD.

**RESULTS**

**Descriptive Statistics**

There was a wide range of sweat gland activation within the images analyzed. The number of glands activated pharmacologically averaged 59 glands/cm², with a SD of 17 glands/cm². The minimum number of glands activated pharmacologically was 22 glands/cm², while the maximum was 99 glands/cm². During exercise in the heat, average sweat gland activation was 81 glands/cm², with a SD of 30 glands/cm². The minimum number of glands activated during exercise was 35 glands/cm², with the maximum observed being 187 glands/cm². As such, the images analyzed provided a good range of sweat gland activation values with which to assess the modified iodine-paper technique with computer-assisted analysis.

**Is There Good Agreement Between Computer and Manual Analysis?**

One-hundred samples were included in the analysis. The number of glands identified with computer-aided analysis (68 ± 29 glands/cm²) did not significantly differ from manual counts (72 ± 24 glands/cm²; \( P = 0.27 \)). The coefficient of variation between manual and computer-assisted counts was 16 ± 15%. The two methods were highly correlated (\( r = 0.77; P < 0.001 \)) and the mean bias ± limits of agreement (computer − manual) was −4 ± 38 glands/cm², with 86% (86 out of 100) of individual differences falling within the qualitative limits of magnitude (Fig. 2A).
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Is Computer-Assisted Analysis Reliable Between Two Independent Investigators?

Three-hundred eighty-two samples were included in the analysis. With the use of computer-assisted analysis, the number of glands identified by investigator 1 (74 ± 28 glands/cm²) did not differ significantly from investigator 2 (70 ± 30 glands/cm²; \( P = 0.07 \)). The coefficient of variation between the two investigators was 7 ± 10%. Counts from both laboratories were highly correlated (\( r = 0.95; P < 0.001 \)), and the mean bias ± limits of agreement (\( \text{analyzer 1} - \text{analyzer 2} \)) was 4 ± 18 glands/cm², with 99% of individual differences (381 out of 382) falling within the qualitative limits of magnitude (Fig. 2B).

What Is the Internal Reliability of the Modified Iodine-Paper Technique?

One-hundred sixty-seven sequential samples were included in the analysis. When duplicate measures were analyzed using computer-assisted analysis, the number of glands in the first sample (73 ± 31 glands/cm²) did not significantly differ from the second sample (71 ± 29 glands/cm²; \( P = 0.37 \)). The coefficient of variation between samples was 11 ± 10%. Sweat gland counts from duplicate samples were highly correlated (\( r = 0.88; P < 0.001 \)), and the mean bias ± limits of agreement (\( \text{sample 1} - \text{sample 2} \)) was 3 ± 29 glands/cm², with 96% (161 out of 167) of individual differences falling within the qualitative limits of magnitude (Fig. 2C). The smallest detectable change calculated from duplicate measurements was 30 glands/cm².

DISCUSSION

The current study examined the agreement between computer-assisted analysis and manual counts of sweat gland activation, the interrater reliability of computer-assisted analysis, as well as the internal reliability of the modified iodine-paper technique. The results demonstrate that computer-assisted counts of active sweat glands show good agreement compared with manual analysis. Further, the number of active sweat glands determined using computer-assisted analysis shows little variability between independent investigators (interrater), as well as between duplicate measurements (internal). Combined with its ease of use, we propose that the modified iodine-paper technique with computer-assisted analysis provides a simple and reliable determination of the number of active sweat glands.

Before the current study, no standard technique to measure sweat gland activation had been advocated. This has led to the use of a variety of techniques, making comparisons difficult and possibly limiting interpretations between different laboratories. Furthermore, no study has specifically examined whether determining the number of active sweat glands is reliable between independent investigators. In the current study, we chose to examine the reliability of the modified iodine-paper technique, with computer-assisted analysis, due in large part to its ease of use and minimal time required for analysis. By examining the reliability of this technique, we were interested in determining whether it could be used as a standard technique to determine the number of active sweat glands.

Is There Good Agreement Between Computer and Manual Analysis?

Computer-determined counts of active sweat glands closely agreed with those obtained by manual count. This was evidenced by a strong correlation and small coefficient of variation between the two methods. Further, the bias and limits of agreement were relatively small between the two methods and fell within the upper and lower ranges of reported differences in the literature (see Fig. 2A). Although the modified-iodine paper technique has previously been validated (31, 32) and used in a number of publications (5, 8, 11, 36, 37), the use of computer-assisted analysis has never been compared with manual counts. It is therefore interesting to compare the counts obtained in the current study with those reported in the literature. The number of active sweat glands measured using the current modified iodine-paper technique provided similar values to those reported in previous studies where manual counts were performed. Specifically, the reported number of active sweat glands during exercise range from ~80 glands/cm² during low (35% \( V_{\text{O}_2\text{max}} \)) to ~120 glands/cm² during moderate (65% \( V_{\text{O}_2\text{max}} \)) intensity exercise in normal ambient conditions (25–30°C, 40–50% relative humidity) up to over 150

Fig. 2. Bland-Altman plot of intermethod (computer vs. manual analysis; A), interinvestigator (investigator 1 vs. 2; B), and internal (sample 1 vs. 2; C) differences in the number of active sweat glands. Circles represent an individual count of active sweat glands (\( A: n = 100; B: n = 382; C: n = 167 \)). In A–C, the bold solid line represents the mean bias, while the dashed lines represent the limits of agreement. Roman numerals represent the largest range of reported differences in sweat gland activation.
glands/cm² during moderate intensity exercise in a hot (35–49°C, 30–80% relative humidity) environment (12, 25, 26, 29). Similarly, the number of active sweat glands during pharmacologically induced sweating range from ~60 to 120 glands/cm² (10, 16). These values resemble the number of active sweat glands measured in the current data set during exercise in the heat (81 ± 30; range of 35–137 glands/cm²) and pharmacological stimulation (59 ± 12; range of 22–99 glands/cm²).

**Is Computer-Assisted Analysis Reliable Between Two Independent Investigators?**

The use of computer-assisted analysis adds a layer of objectivity to the determination of the number of active sweat glands for a given sample. When manually counting the number of sweat glands, the investigator must subjectively determine dots that actually constitute a single sweat gland vs. those that may have converged due to a high output from multiple glands. In contrast, the use of computer software allows the user to define a lower and upper size limit for the pixel area, which is the minimum/maximum size allowable for a dot to be considered in the count. It is important to note that using a fixed pixel range has the potential to reduce all subjectivity associated with the determination of what constitutes a gland or not when analyzing multiple samples. However, this approach assumes that the sweat glands from all collected samples will be of the same size. This may very well occur when measurements are taken from a given anatomical location during a repeated measures design experiment (e.g., pre- to postacclimation on the forearm). In the current study, however, the use of a fixed pixel range limit was deemed inappropriate as the anatomical locations (e.g., back vs. forearm) and populations (males vs. females) from which the samples were obtained resulted in different gland sizes on the collected samples. Consequently, the investigators freely adjusted the upper and lower pixel size limits based on a subjective interpretation of what should be considered a sweat gland or not. However, adjusting the upper and lower size limits does add subjectivity to the computer-aided analysis, which led us to examine interrater reliability. Even though the investigators were allowed to determine the pixel area limits, and therefore the size of dots that would be considered a gland or not, there was little interrater variability for individual counts. There was a very high correlation between analyzers ($r = 0.95$), and the mean bias between investigators was only 4 glands/cm², with all but one individual difference falling inside the limits of magnitude (see Fig. 2B). These results favor the use of computer-assisted analysis as a standard procedure to determine the number of active sweat glands between independent laboratories.

**What Is the Internal Reliability of The Modified Iodine-Paper Technique?**

The results from the current study suggest there is some variability between duplicate measurements, although with no evident systematic bias between the first and second measurements (Fig. 2C). It is important to note that the variability in the number of active sweat glands over duplicate measurements was due to variability in the modified iodine-paper technique itself, as the counts were performed by computer-assisted analysis. To our knowledge, no study has specifically examined the internal reliability of determining the number of active sweat glands, regardless of the method employed. However, Buono and Sjoholm (8) did report a 5% coefficient of intrasubject variation when using the modified-iodine paper technique combined with manual analysis. In contrast, we report an intrasubject coefficient of variation of $11 \pm 10%$. Furthermore, the smallest difference worth detecting calculated from duplicate measurements was 30 glands/cm², suggesting that differences in sweat gland activation of less than ~30 glands/cm² using the current method may not be worth considering (i.e., differences within the error of the measurement). Together, these values provide a basis to determine whether observed differences in sweat gland activation should be interpreted as meaningful when using the evaluated techniques. It is interesting to note that the calculated smallest detectable change is consistent with previous studies (5, 8, 14, 18, 26) reporting significant differences in sweat gland activation between various populations and/or experimental conditions. Duplicate measures were also highly correlated ($r = 0.88; P < 0.001$) and the mean bias ± limits of agreement (sample 1 – sample 2) was $3 \pm 29$ glands/cm², with the vast majority of individual differences falling within the limits of magnitude (Fig. 2C). These data indicate that the modified-iodine paper technique with computer-assisted analysis is sensitive enough to detect typical changes in sweat gland activation.

**Perspectives**

The use of standard measurement techniques and analytical tools is important to ensure consistency and allow for more direct comparisons between independent laboratories. In the current study, we used the modified iodine-paper technique due to its ease of use and minimal need of equipment. It also allows for a rapid determination of the number of active sweat glands and thus multiple measurements at a given time period, as opposed to relying on a single sample. While we did not compare the current method with those of previous studies (e.g., starch-iodine and mattraphotographic), the results of the current study provide a strong basis for advocating the use of the modified-iodine paper technique combined with computer-assisted analysis for the standard determination of sweat gland activation. First, computer-assisted counts of the number of active sweat glands show good agreement with those obtained manually. The advantages of computer-assisted analysis are that it provides a more objective count of the number of active sweat glands, and the analysis of a sample is done within minutes, as opposed to longer periods of time required for manual analysis. Second, computer-assisted analysis is reliable between two investigators from independent laboratories. This ensures that the counts obtained in one laboratory can be reliably replicated in another, making comparisons between different laboratories more direct and meaningful. Third, the measurement technique itself shows little variability between duplicate measures, resulting in relatively low values of measurement noise and of smallest detectable difference. As such, small differences in sweat gland activation between populations and/or experimental conditions can be identified.
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Conclusion

The current study examined the modified iodine-paper technique with computer-assisted analysis for the simple and reliable determination of sweat gland activation. Computer-assisted counts of active glands showed good agreement compared with commonly used manual counts. Furthermore, computer-assisted determination of active sweat glands showed little interrater variability. Finally, we examined the internal reliability of duplicate measurements of sweat gland activation, which fell within the range of previously reported differences. Based on the observed results, we propose that the modified-iodine paper technique with computer-assisted analysis be employed as a standard measurement of sweat gland activation.

APPENDIX

Using ImageJ to Count the Number of Active Sweat Glands

Step 1) to analyze a sample, determine the edges of the scanned image using the “Find edges” option (Process toolbar–Find edges).

Step 2) next, set the image type to 8-bit grayscale (Image toolbar–Type–8-bit) before converting the image to a binary (black and white) image (Process toolbar–Binary–Make binary). Once this step is complete, the dots produced by the active glands are displayed in black, with the background being white.

Step 3) to perform a count of the number of active glands, select the “Analyze particles” (Analyze toolbar–Analyze particles and first define a lower and upper size limit for the pixel area, which is the minimum/maximum size allowable for a dot to be considered in the count. Before running the analysis, ensure that the following options are chosen: display results, clear results, exclude on edges, record results. Also, ensure that the “Outlines” option is selected under the “Show” menu to visually examine which particles have been included by the software during the analysis.

Step 4) once the analysis performed, the software generates a count of the particles present in the image, which is the number of active glands for that sample. It also provides an image in which each individual count included in the analysis has been circled in red. If the number of particles included in the analysis is inadequate, return to step 3 and adjust the lower and upper size limit of the pixel area accordingly.

Step 5) the number of particles is divided by the surface area of the paper to give a value of active sweat glands per square centimeter.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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