Efficient Stool Collection Methods for Evaluating the Diarrhea Score in Mouse Diarrhea Models

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Abstract. Background/Aim: The mouse diarrhea score is usually determined by evaluating stool consistency and shape. Thus, defecated stools should be collected without damage or contamination. The study aimed to develop improved mouse stool collection methods and diarrhea-scoring criteria. Materials and Methods: We developed improved stool collection methods (paper towel methods) and compared them with previously used ones (stool collection using regular cages containing bedding chips or filter paper and metabolic cages). Results: Compared to previously used methods, paper towel methods collected stools without bedding-chips-induced contamination, mouse body/foot-induced damage, or sampling errors. When using paper towel methods, wet stools create water marks (diarrhea marks) on paper towels with strong water absorption capacity, by which diarrheal severity can be analyzed semi-quantitatively. To improve the objectivity in determining diarrhea scores, practical diarrhea-scoring criteria were also proposed. Conclusion: These results would be helpful to researchers facing difficulties in evaluating the mouse diarrhea score.

Mouse models have been used to study various diarrheal diseases (1-69). In many studies employing mouse diarrhea models, the diarrhea score is usually determined on the defecated stool to compare the severity of diarrhea between experimental groups (1-66). The diarrhea score is determined by scoring various parameters graded in 2-5 steps according to the severity of diarrhea (1-69). Parameters frequently employed in evaluating the diarrhea score were stool consistency, stool stickiness, stool water content, stool shape, the presence of mucus or blood, diarrhea-induced fur staining, and body weight reduction (1-69).

All parameters described above, except for diarrhea-induced fur staining and body weight reduction, can only be evaluated when feces are properly collected. To correctly evaluate the severity of diarrhea on the stool, defecated feces should be collected without damage caused by external factors such as contamination by bedding chips or crushing by being stepped on by the mouse foot and body.

We recently evaluated the diarrhea score in a study using a mouse model of 5-fluorouracil (5-FU)-induced diarrhea (70). When we tried to collect feces during the study, we encountered difficulties in collecting intact feces by using regular mouse cages containing bedding chips, because many defecated feces were contaminated or damaged by the bedding chips or the foot and body of the moving mice. It was also difficult to evaluate the diarrhea score on the stool stuck to the bedding chips. Moreover, it was often difficult to determine the exact grade for the parameters employed in evaluating the diarrhea score, because of the overlapping ambiguous boundaries between grades described in many previous studies (2, 5, 6, 17, 22).

In the present study, we searched the literature published in the last five years reporting the results of the mouse diarrhea score and identified the drawbacks of the previously used stool collection methods and criteria for determining the diarrhea score. Consequently, in this study, we aimed to propose improved methods for stool collection (paper towel methods which can collect defecated stools with little damage). More practical criteria and procedures for grading the diarrhea score on defecated stools are also proposed with clear boundaries between grades. The results can be helpful to researchers who encounter difficulties in evaluating the severity of diarrhea in experimental mouse models.
Materials and Methods

**Mice.** Specific pathogen-free BALB/c mice (6-8 weeks of age) were purchased (Damul Experimental Animal Center, Daejeon, Republic of Korea) and housed in steel wire net-covered plastic cages containing bedding chips (Damul Experimental Animal Center) (regular cage) at the Jeonbuk National University Hospital Animal Care Facility. Mice were maintained under a 12 h dark-light cycle at a temperature of 20-22°C (45-70% humidity) and fed on food and water *ad libitum*. Mice were age- and sex-matched at the onset of each experiment. Experimental protocols were approved by the Jeonbuk National University Animal Care and Use Committee (JBNU 2020-047).

**Mouse diarrhea models.** We employed two types of mouse diarrhea models, the 5-FU-induced diarrhea model and the castor oil-induced diarrhea model. These two models have different characteristics of diarrhea. Compared with the 5-FU-induced diarrhea model, the castor oil-induced diarrhea model has the characteristics of faster induction of diarrhea, shorter duration of diarrhea, and more frequent watery diarrhea. By employing two different diarrhea models, the present study attempted to increase the potential that the experimental results could be applied to different mouse diarrhea models having various diarrheal characteristics.

In the 5-FU-induced diarrhea model, 5-FU (Ildong Pharmaceutical Co., Seoul, Republic of Korea) was intraperitoneally injected at a dose of 30 mg/kg body weight daily for 5 consecutive days (days 1-5). In the castor oil-induced diarrhea model, castor oil (Merck, Seoul, Republic of Korea) was administered at a dose of 20 ml/kg body weight as a single dose by using oral gavage.

**Comparison of stool collection methods.** The stool collection methods employed in searched studies and the “paper towel method” devised by the authors were compared in terms of the degree of damage to the stools collected. In some experiments, differences in convenience between the stool collection methods were also checked.

**Comparison of water marks.** Using a micropipette, aliquots of 20 μl of water were instilled in triplicate on the paper towel (Yuhan-Kimberly, Seoul, Republic of Korea) and the filter paper (Sartorius Stedim Biotech, Seoul, Republic of Korea). The longest diameter of the water mark generated on them by the instilled water was measured after 2 min.

**Statistical analysis.** Data are expressed as mean±standard deviation (SD). Differences between two groups were analyzed by Student’s *t*-test. Data were analyzed with SPSS statistics 23 (IBM, USA) and graphed using Microsoft Excel 2010 (Microsoft, USA). *p*<0.05 (two-sided tests) was considered statistically significant.

Results

**Feces collection methods employed in the searched literature.** Most studies did not have specific descriptions of the feces collection method, and were considered to have collected feces in regular cages containing bedding chips. There were only 7 studies that described the feces collection method (Table 1). In three of the seven studies, a filter paper was placed on the cage floor without bedding chips to collect stools, and the severity of diarrhea was determined by measuring the size of the diarrhea marks formed on the filter paper (20, 63, 64). In one study, the bedding chips of the cage were covered with white paper towels to observe the bloody feces (65). In another study, feces were collected using a metabolic cage (62), and in the other two studies, the severity of diarrhea was evaluated on stools defecated while holding the mouse in the hand (19, 66).

Among the feces collection methods searched from the literature, 1) one using bedding chips-containing regular cages, 2) one using regular cages having a paper towel on the floor instead of bedding chips (As a representative method of one using cages having a filter paper on the cage floor without bedding chips and one using regular cages having a paper towel on the bedding chips), and 3) one using metabolic cages, were compared with the “paper towel method” devised by the authors in terms of the capability of collecting intact stools without damage. The method evaluating defecated feces while holding the mouse in the hand was excluded from the comparison experiments because it was considered unsuitable for its use in usual experiments dealing with a large number of mice.

**Parameters employed in evaluating the diarrhea score in the searched literature.** The parameters for evaluating the severity of diarrhea employed in the searched studies were stool consistency, stool stickiness, stool wetness (stool water content), stool shape, and the presence of occult or gross blood and mucus (1-66). In some studies, the severity of diarrhea was evaluated by observing the size of fur coat staining caused by diarrhea (3, 6, 8).

Parameters such as stool consistency, stool stickiness, stool wetness (stool water content), and stool shape are simultaneously affected by the water content of the stool. All these parameters were considered to be the same even though they have different expressing terms. “Stool consistency” was therefore employed as the term representing all these parameters unless otherwise noted.

**Paper towel method.** To correctly evaluate the severity of diarrhea on feces, feces should be observed without being
damaged by external factors such as bedding chips or mouse foot and body. Since many studies employed stool consistency (closely related to stool wetness or water content) as an important parameter for evaluating the diarrhea score, collecting feces on a paper towel with good water absorption capability would be useful. In other words, if the water mark (diarrhea mark) generated on the paper towel by the wetness of stools was observed, it would be more objective than judging the wetness on the stool itself with the naked eyes only. The paper towel method was therefore devised as a method for collecting feces with the merits (Figure 1A). The paper towel method was performed as follows: 1) Place paper towels on the experiment table. 2) Put the steel wire net floor (the bottom part of the mouse-dwelling portion of the metabolic cage, Nalgene, Rochester, NY, USA) upside down on the paper towels. This creates a gap of about 2 cm between the steel wire net and the paper towels. Therefore, defecated stools dropped on the paper towel can be evaluated without damages caused by being stepped on by the mouse foot or body. 3) Place the upper plastic cage (the upper plastic part of the mouse-dwelling portion of the metabolic cage) on the steel wire net floor. 4) Place mice in the cage and evaluate defecated stools seated on the paper towel by being dropped down through the steel wire net for required periods. Note water marks or diarrhea marks (thick arrows) generated on the paper towel by wet stools collected from mice of the 5-FU-induced diarrhea model on day 11 (B).

The size of water absorption marks (water marks) on the paper towel vs. the filter paper. In some previous studies, the severity of diarrhea was evaluated by collecting feces on the filter paper and comparing the size of the water mark (diarrhea mark) generated on the filter paper by absorbing water from wet feces (20, 63, 64). We evaluated whether the paper towel generates larger or better evaluable water absorption marks (water marks) than the filter paper. The longest diameter of the water marks generated on the paper towel (2.3±0.1 cm) was larger than that on the filter paper (1.6±0.0 cm, p<0.001) (Figure 2). This result led us to use the paper towel instead of the filter paper in the paper towel method.

The stool collection method using regular cages vs. the paper towel method. In a regular cage containing four mice of the 5-FU-induced diarrhea model, the bedding chips were replaced with new ones on day 8, and defecated feces were observed after 1 h. As shown in Figure 3A, many feces stuck to the bedding chips. Although the feces sticking to the bedding chips were judged to be diarrheal ones, stool consistency and shape were hard to be determined due to the bedding chips stuck to the feces. On the other hand, when the defecated feces were collected for 1 h using the paper towel method, most of them were evaluable for the shape as well as the consistency/wetness (Figure 3B). In particular, the consistency/wetness of the stool was easily identified due to the water mark generated on the paper towel by the moisture in the stool (arrows in Figure 3B).

The stool collection method using cages having the paper towel placed on its floor vs. the paper towel method. In a regular cage containing four mice of the 5-FU-induced diarrhea model, the bedding chips were removed on day 8, and a paper towel was placed on the cage floor, and defecated feces were observed after 1 h. As shown in Figure 4A, it was hard to determine the shape and consistency of
the feces because the feces were crushed by being stepped on by the foot and body of the mice. In contrast, when feces were collected for 1 h using the paper towel method, the consistency/wetness and shape of the feces could be determined with fewer difficulties (Figure 4B).

The stool collection method using the fully equipped metabolic cage vs. the paper towel method. Four mice were placed in a fully equipped metabolic cage 1 h after the oral administration of castor oil and feces were collected for 2 h. As shown in Figure 5, only a small fraction of defecated feces which have normal shapes and low stickiness were collected in the feces collection tube, and the remained large fraction of defecated feces were stuck on the sliding down side wall of the metabolic cage. Therefore, the evaluation of the feces was difficult when using the fully equipped metabolic cage compared with when using the paper towel method. It was also more inconvenient than the paper towel method because it took more time to assemble/disassemble and clean the parts of the cage. Besides, not only a small fraction of feces were collected in the feces collection tube, but it was also difficult to evaluate feces in situ within the tube. Therefore, the feces must be moved out of the tube for evaluation. The feces could be damaged during the moving out process, especially when the feces were sticky. On the other hand, when the paper towel method (using only the mouse-dwelling portion of the metabolic cage) was employed, the shape and consistency/wetness of the feces could be determined with fewer difficulties.

Modification of the paper towel method. The paper towel method requires the mouse-dwelling portion of the metabolic cage including a steel wire net (although not a fully equipped metabolic cage) (Figure 6A). However, there are laboratories that do not have metabolic cages. For such laboratories, two modified paper towel methods were devised in the present

Figure 2. Differences in the size of water marks generated by the instilled water between the paper towel and the filter paper. Two minutes after instilling aliquots of 20 µl of water on the paper towel (A, upper) and the filter paper (A, lower), the longest diameter of the water marks generated on them was measured. A representative picture (A) and mean±SD of the sizes of the water marks (B) are presented. ***p<0.001.

Figure 3. Stools collected by using the regular cage containing bedding chips (A) vs. the paper towel method (B). Defecated stools were collected from mice of the 5-FU-induced diarrhea model on day 8. Note water marks or diarrhea marks (red arrows) generated on the paper towel by wet stools (B).
First, the regular cage was placed upside down on pedestals (Two test tube racks were employed as pedestals) laid on the paper towel (Figure 6B). Second, only the plastic part of the regular cage was placed upside down on a steel wire net (gridiron, etc.) put on pedestals (Two test tube racks were employed as pedestals) laid on the paper towel (Figure 6C).

Defecated feces were collected and observed by using the two modified paper towel methods. The paper towel method served as a control. Four mice of the 5-FU-induced diarrhea model were employed for each method on day 9. The two modified paper towel methods showed comparable performance in evaluating stools to the paper towel method in terms of the capability of collecting intact stools without damage (Figure 6D-F).

**Discussion**

In studies employing mouse diarrhea models, the severity of diarrhea was assessed by using a variety of diarrhea scores (1-69). The parameters for evaluating the diarrhea scores were stool consistency (wetness, stickiness, water content), stool shape, and
the presence of blood (gross or occult blood) and mucus in the stool (1-69). To correctly evaluate these parameters, defecated stools should be collected without damage.

The feces collection methods used in the searched studies were as follows: A) feces collection using the regular cage containing bedding chips (studies without specific descriptions about the feces collection method), B) feces collection on the filter paper placed on the regular cage floor without bedding chips (20, 63, 64), C) feces collection on the paper towel placed on the bedding chips in the regular cage (65), D) feces collection using the metabolic cage (62), and E) feces collection while holding the mouse in the hand (19, 66).

In the present study, the paper towel method devised by the authors was compared with the above-described stool collection methods in terms of the capability of collecting intact stools without damage. Feces collection while holding the mouse in the hand was excluded from the comparison experiments because it was considered inadequate for use in usual experiments dealing with a large number of mice.

When collecting feces by using the regular cage containing bedding chips (A), wet stools were stuck to the bedding chips and damaged. Therefore, it was hard to determine the stool consistency and shape, which are the parameters commonly employed in evaluating the diarrhea score.

Feces collection on the filter paper placed on the regular cage floor without bedding chips (B) and feces collection on the paper towel placed on the bedding chips in the regular cage (C) were considered to be similar methods. Therefore, only the method having a paper towel laid on the cage floor without bedding chips was tested as the representative of the two methods. It was also hard to evaluate the consistency and shape of the feces because of the fecal damage caused by being stepped on by the mouse foot and body.

In feces collection using the fully equipped metabolic cage, only a small fraction of defecated feces having low stickiness (wetness) were collected in the feces collection tube, and the remaining large fraction of feces were stuck to the sliding down side wall of the metabolic cage. The feces collected in the feces collection tube have to be transferred onto a paper towel to assess the diarrhea score. During the transferring process, the feces may be damaged, and therefore unfit for evaluating their consistency and shape, especially when they were entangled together in the small space of the feces collection tube.

On the other hand, the paper towel method collected the feces with little damage compared to the searched feces collection methods. The paper towel method was therefore considered efficient and convenient to evaluate the parameters employed in determining diarrhea scores.

Some previous studies evaluated the size of the water mark (diarrhea mark) generated on the filter paper as a parameter to determine the diarrhea score (20, 63, 64).
However, the size of the water mark generated on the paper towel by the same amount of instilled water was larger than that on the filter paper in the present study. This result indicated that the paper towel is more sensitive than the filter paper in detecting the water (wetness or moisture) contained in the stool. This result led us to employ the paper towel instead of the filter paper as a component of the paper towel method. The paper towel is also cheaper than the filter paper. The paper towel method can evaluate the size of the water mark as well as its presence generated by the wet stool. Therefore, the paper towel method can be more sensitive and objective than evaluating the stool consistency/wetness with the naked eyes only or by using the filter paper. These findings indicated that the paper towel method can be preferred when evaluating the stool consistency/wetness.

The paper towel method requires the mouse-dwelling portion of the metabolic cage. However, there are laboratories that do not have metabolic cages. For such laboratories, two modified paper towel methods were devised in the present study. The modified methods no longer require the mouse-dwelling portion of the metabolic cage. They showed to have comparable stool collection functions to the paper towel method in terms of the capability of collecting intact stools without damage.

Many previous studies did not describe feces collection methods employed in evaluating the diarrhea score. They were considered to have used regular cages containing bedding chips in collecting defecated feces. Regardless of the presence of the description, searched feces collection methods did not seem that practical, as confirmed in the present study. Researchers using mouse diarrhea models for the first time may therefore encounter difficulties in collecting intact stools and consequently in evaluating the diarrhea score. Moreover, the grading boundaries of various diarrhea scores are ambiguous, adding more difficulties in evaluating the diarrhea score (2, 5, 6, 17, 22). This study was intended to be helpful in those cases.

The most accurate way to assess the diarrhea score would be to monitor the mouse continuously, detect when it is defecating, and evaluate the defecated stool immediately, e.g., the evaluation of the stool being defecated while holding the mouse in the hand (19, 66). However, considering that most mouse experiments do not deal with a small number of mice, but a large number of mice, it is not that practical. Another accurate way would be to evaluate the stool in the rectum or the large bowel after sacrificing the mouse (67-69). However, it can only evaluate the diarrhea score once per mouse, and serial evaluations over several days are difficult. There is also the inconvenience of sacrificing the mouse each time for the stool evaluation. Therefore, the paper towel method or modified paper towel methods can be recommended in experiments evaluating the diarrhea score, because feces can be collected for required periods without such inconvenience.

In the searched literature, the number of grades of various diarrhea scores determining the degree of stool consistency ranges from 2 to 5. Examples according to the number of grades are as follows: 2-grade method (normal stool vs. diarrhea stool), 3-grade method (normal stool vs. soft stool vs. watery or very soft stool), 4-grade method (normal stool vs. wet stool vs. loose stool vs. watery stool), and 5-grade method (normal stool vs. slightly loose stool vs. loose stool vs. watery stool vs. severe diarrhea). The more the number of grades, the more ambiguous the boundaries between grades. Therefore, it may be hard to obtain reproducible diarrhea scores. Thus, there were studies in which two or more researchers evaluated the diarrhea score blindly for the same stool sample (19, 52, 54, 68, 69). However, the attempt does not seem efficient.

Therefore, a new grading method was proposed to obtain a more objective diarrhea score in the present study. As the number of grades that distinguish stool consistency increases, the boundaries between grades become more
ambiguous. The proposal of the 4-grade method or 5-grade method was therefore excluded. Considering a 3-grade method first, the stool consistency can be divided into normal stool, wet stool, and watery stool. A practical procedure for determining the diarrhea score by employing this 3-grade method is proposed as follows: 1) Collect feces using the paper towel method or modified paper towel methods. The duration of stool collection can be determined depending on the frequency of defeation of the employed mouse model. For example, in the present study, feces were collected for 1 h in the 5-FU-induced diarrhea model and 2 h in the castor oil-induced diarrhea model. 2) Take a photograph of the paper towel on which the feces are seated. 3) Determine the stool consistency grade by observing the photograph. The definition of each grade of stool consistency proposed by the authors is as follows (Figure 7): grade 1/normal stool (normal shaped stools without water mark on the paper towel), grade 2/wet stool (normal shaped stools with water mark beyond the edge of the stool on the paper towel), and grade 3/watery stool (unshaped stools with water mark beyond the edge of the stool on the paper towel or watery diarrhea with scantly formed components). In many previous studies, grade 2 or more was regarded as diarrhea (1-69). Researchers who want to employ the 2-grade method can evaluate the stool as follows: grade 1 (normal stool) and grade 2 (wet stool or watery stool).

When grading the stool consistency, one of the two methods (2- and 3-grade methods) can be selected according to the diarrhea pattern of the mouse model employed in each study. Of course, this proposal requires more validation, and the definition and number of grades can be modified according to the characteristics of each study.

In some studies, the diarrhea score was determined by observing the severity of the fur coat staining caused by diarrheal stools (3, 6, 8, 35, 60). In many previous studies, the disease activity index (DAI) was employed to evaluate the severity of diarrhea (2, 9, 17, 24, 33). DAI is calculated by scoring the grade of the three parameters (stool consistency, stool blood, and body weight) and summing these scores. In some studies, only two of the three parameters, stool consistency and stool blood, were evaluated to determine DAI (37, 40). In other studies, the diarrhea index (DI) was employed to evaluate the severity of diarrhea (20, 63, 64). DI was calculated by multiplying the diarrhea incidence rate (the number of diarrheal feces + the total number of feces) by the mean loose stool grade. Fecal water content (wet weight – dry weight) (12, 49) and fecal friability tested by pressing with tweezers were also used to evaluate the stool consistency (1, 5).

**Conclusion**

In the present study, the paper towel method and the two modified paper towel methods were devised to collect mouse feces without damage. New modified procedures for evaluating the mouse diarrhea score were also proposed to help researchers who face difficulties in determining the diarrhea score. The results of the present study may be applied to studies employing rat diarrhea models and to studies requiring intact defecated stools with little damage.

**Conflicts of Interest**

The Authors have no conflicts of interest to declare in relation to this study.

**Authors’ Contributions**

S.K.Y., S.W.K., and S.T.L. conceived and designed the experiments. S.K.Y. performed the experiments. S.K.Y., S.W.K., and S.T.L. analyzed the data and wrote the article.

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