Effects of Nesting Material on the Toxicologic Assessment of Cyclophosphamide in Crl:CD1(ICR) Mice

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The provision of nesting material benefits mice by reducing cold stress, improving feed conversion, increasing litter size, and improving adaptive immunity. The effects of toxins are sensitive to environmental changes, and the introduction of novel items can alter results in some toxicologic studies. We hypothesized that nesting material would reduce stress and positively alter immunologic parameters in Crl:CD1(ICR) mice, thus changing typical results from a well-studied immunomodulating drug, cyclophosphamide. A 13-wk study assessed the following treatments in a factorial design ($n = 4$; 32 cages total): nesting (0 or 10 g) and drug (50 mg/kg cyclophosphamide or 10 mL/kg saline; IP weekly). Detailed examinations and body weights were recorded weekly, and nests were scored twice weekly. Fecal pellets were collected at 0, 4, 6, and 12 wk for analysis of corticosterone metabolites. At study termination, clinical pathology and immune parameters were collected, a necropsy performed, and lymphoid organs and adrenal glands were submitted for histopathology. All expected results due to cyclophosphamide were observed. Nesting reduced the proportion of mice with piloerection, and body weights were highest in saline-nested male mice. No differences in hematology, clinical chemistry, or absolute lymphocyte counts were observed. Corticosterone metabolites in all nested groups were not different from baseline levels but all nonnested groups had higher levels than baseline. Nested cyclophosphamide-treated groups had significantly lower corticosterone levels than nonnested cyclophosphamide-treated groups. This study illustrates that nesting material does not alter the results of a standard toxicology study of cyclophosphamide but alleviates study-related stress and improves mouse welfare.

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Mouse housing has changed substantially over the last 10 y. The widespread acceptance and incorporation of environmental enrichment (for example, nesting material, huts, chewing items), development of new bedding materials, and ventilated caging are examples of these changes. Nesting material for mice, in particular, is now used much more widely in the United States, is required in the United Kingdom, and is highly recommended in Canada. This change is likely the result of the accumulation of literature demonstrating the benefits of nesting material on mouse physiology and wellbeing.^{13,16} Consequently, the scientific community has begun to perceive the provision of nesting material to mice not only as an efficient and effective environmental enrichment but also as an important basic physiologic need. Although nesting material is often provided, many researchers may choose to not use the material because they do not know how it will affect their research or whether it will cause increased variability.²⁷ This is especially true in fields of study that are sensitive to environmental influences, such as toxicology. Toxicologic screening is a critical step in the process of drug approval through regulatory agencies prior to clinical trials in humans. Therefore, understanding the effect of environmental enrichment on toxicologic study endpoints is

crucial, because this practice might affect the safety assessment of potential medications. A previous experiment demonstrated that the provision of wood blocks to rats did not alter the results of a typical exploratory short-term study (28 d).⁹ In addition, recent evidence suggests that the provision of nesting material to naïve mice for 13 wk had no clinical impact on common toxicologic measurements.⁸ However, the effect of enrichment on long-term toxicology studies has not yet been determined, and the use of nesting material will not likely become widely accepted until its effects on scientific outcomes and interaction with test compounds are known.

The provision of environmental enrichment provides animals with stimuli or substrates that address a behavioral need. Ultimately enrichment should facilitate homeostatic goals of behavior and shield animals from stressors.⁵⁰ Nesting material, unlike other forms of enrichment, has been shown to meet these criteria for mice.^{12,13,22} Mice show a strong drive to collect and move nesting material even when housed in temperatures at which the thermal insulation from the nest is not needed.^{12,42} Therefore, the provision of materials that allow mice to express this behavioral need and achieve a behavioral goal, a nest, is therefore enriching. Not only is nesting material beneficial by increasing natural behaviors but it has a positive effect on mouse physiology. Mice housed at standard laboratory temperatures (20 to 26 °C) are cold-stressed¹⁸ and must use additional energy to stay warm.^{6,11,35,37} Recently, mice housed at standard laboratory temperatures have been shown to have increased tumor growth and reduced adaptive immunity compared with those housed in thermoneutrality (approximately 30 °C),^{25,33} thus

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indicating the effect of cold stress on immune function. However, 8 to 10 g of nesting material added to mouse cages reduces heat loss and physiologic thermogenesis,¹³ improves feed conversion¹³ and reproductive performance,^{16,17} and increases energy balance during inactivity.²⁹ Furthermore, alteration in nest building is a robust behavioral indicator of the animal's overall wellbeing. When mice are in pain or experience a general malaise, nest-building behavior decreases, affecting nest shape.^{15,28}

The goal of the current experiment was to determine whether the provision of nesting material would affect results from a known toxicologic agent. Cyclophosphamide was chosen for its well-characterized effect on the immune system and for its induction of well-documented clinical signs in the mice. We hypothesized that nesting material would reduce stress and would positively alter immunologic parameters, thus changing the toxicologic results from a well-studied immunomodulating drug. We predicted that the provision of nesting material to mice for 13 wk would have an effect on stress, thus altering immunophenotyping measures typical of a well-studied immune-modulating compound, such as cyclophosphamide.

Materials and Methods

Animals and housing. All work was conducted at Charles River Laboratories' AAALAC-accredited facility in Quebec, Canada, and was approved by the Preclinical Services Montreal's IACUC (protocol no. 14102). All work conducted in this study is compliant with the *Guiding Principles in the Use of Animals in Toxicology Studies*.⁴⁴ At the start of the study, all mice tested negative for a list of common mouse infectious agents.⁷ No further health monitoring was performed.

A total of 48 male and 48 female Crl:CD1(ICR) mice were received from Charles River Canada (Saint-Constant, Quebec, Canada). This strain was chosen because it is the most commonly used and a well-characterized strain in preclinical research. On arrival, the 4-wk old mice were socially housed (3 mice per cage) in conventional solid-bottom polycarbonate cages (6.75 × 12 × 7.5 in.; One Cage, Lab Products, Seaford, DE) containing bedding (Bed-O'Cobs, The Andersons Lab Bedding, Maumee, OH) and equipped with an automatic watering valve. Mice were obtained and grouped prior to puberty to minimize aggression⁴⁶ and were given 4 wk prior to the start of the experimentation to acclimate to their new environment. On arrival, cages were randomly assigned to their respective treatment groups by using inhouse proprietary software (Statistical Reporting System version 1.4). Male and female cages were randomized separately. After randomization, half of the cages were provided with 10 g of nesting material (Enviro-Dri, Shepherd Specialty Papers, Watertown, TN). This quantity was more than the recommended minimum of 8 g,¹² because the literature indicates that females may still experience some cold stress at this level.^{12,13} A small amount of the old nesting material was transferred to the new cage at cage change, when dry and fluffy. The remaining soiled nesting material was weighed, discarded, and replaced by an equivalent amount in weight of new nesting material. Each mouse was identified by using a tail tattoo system (AIMS, Hornell, NY). Mice remained group-housed for the duration of the study. A commercial chow (14% protein; PMI Nutrition International Certified Rodent Chow no. 5CR4, LabDiet, St Louis, MO) was provided without restriction throughout the study. The mice were kept on a 12 h:12 h light:dark photoperiod (lights on, 0700), in a room set to 22 ± 3 °C with a targeted relative humidity of 30% to 60%. To minimize aggression between conspecifics, excessive animal manipulation, cage disturbance,

and foreign olfactory cues were avoided. To minimize olfactory cues, the only surfaces that mice contacted were their home cages and technicians' gloved hands; technicians changed gloves whenever they became soiled with urine.

Experimental design. Nesting material (0 or 10 g) and drug (cyclophosphamide or saline) administration were the 2 main treatments applied in a 2×2 factorial design, resulting in 4 treatment groups: group 1, 0 g nesting material plus saline (nonnested saline-treated); group 2, 10 g nesting material plus saline (nested saline-treated); group 3, 0 g nesting material plus cyclophosphamide (nonnested cyclophosphamide-treated); and group 4, 10 g nesting material plus cyclophosphamide (nested cyclophosphamide-treated). Groups 3 and 4 received weekly injections of cyclophosphamide monohydrate (10 mL/kg IP; 50 mg/kg, Sigma-Aldrich, St Louis, MO) for 13 wk; mice of groups 1 and 2 were treated with an equivalent amount of saline (0.9% sodium chloride for injection, USP, Baxter, Mississauga, Ontario, Canada). The intraperitoneal route was selected as it is a well-characterized administration route for cyclophosphamide in mice.^{24,31} The dose level was selected based on information from the literature and was expected to produce minimal to moderate toxic effects and clinical signs to characterize the potential effect of nesting.^{24,31,40,47} The dose volumes were based on the most recent body weight measurement. The first day of dosing was designated as day 1.

Clinical observations. Weekly beginning at week -1, mice were removed from the cage for a detailed examination and determination of individual body weight. The examination included a cageside observation of behavior, general condition, and respiration prior to removal from the cage. In addition, the cage environment was examined for the presence and consistency of feces or any abnormal discharge, such as blood. A complete physical examination was performed, including assessment of extremities, eyes, nares, ears, mouth, teeth, skin, and fur and abdominal palpation. Because the nest was disturbed during these assessments, mice were examined and weighed after nest scoring, at 1500 (± 1 h). Location of mice in regard to their nest was documented as 'in the nest site,' 'out of the nest site,' or 'location of the nest site unknown.' For mice not provided with nesting material (groups 1 and 3), the nest site was determined as the area where mice were sleeping. If mice were not sleeping, the nest site was considered as a cup-shaped indentation in the bedding. The technicians and animal caretakers were not blinded to any of the treatments.

Beginning on day -7 until study termination, mice were observed daily (except on days on which detailed examinations were performed) at 1500 (± 1 h). Mice were not removed from their cages during observation, unless necessary for identification or confirmation of observations. When a mouse looked ill or showed clinical signs, its location in regards to the nest (in or out of the nest) was documented, and an unscheduled detailed examination was performed.

Food consumption was measured quantitatively weekly, starting at week -1 and continuing throughout the study. At the beginning of each weekly interval, full food hoppers were weighed. At the end of the interval, the remaining food and hoppers were weighed; hoppers were then refilled and reweighed to start the next interval. The difference between the starting food weight and that remaining represented the total amount of food consumed by all mice of a given cage during 1 wk. This amount was divided by the number of mice in the cage to provide the average food consumption per mouse (a cage-level average) and by 7 to get the average daily consumption. Throughout the study, some mice ground their food, resulting

in considerable food waste (that is, orts) on the bottom of the cage.⁴¹ This quantity was defined as ‘spillage,’ and food consumption was not recorded those weeks because it could not be determined accurately.

Nest scoring. Nest scoring was performed twice weekly starting on day –4 and continued throughout the 13-wk study. On the basis of known pharmacology of cyclophosphamide, we expected the onset of clinical signs at 3 to 4 d after injection.^{24,40} Therefore, nest scoring was performed before and at 3 d after dosing. Scores were recorded between 1300 and 1400 according to a previously published protocol.^{14,23} In brief, each of the 4 walls of the nest was scored according to a scale of 0 to 5; these 4 scores were averaged together to provide the overall cage nest score. A score of 0 was given to cages where nesting material was present but left undisturbed; a score of 1 represented a cage where the nesting material was manipulated, but no nest site could be identified; a score of 2 indicated a flat nest; 3 was a cup nest; 4 was an incomplete dome; and 5 was a complete and enclosed dome. Mice readily use bedding material as nesting substrate, especially when no other material is provided specifically for this purpose.¹² Therefore, nest scoring was performed for all cages, including those not provided with nesting material (groups 1 and 3). Nest scores were averaged by week.

Fecal corticosterone metabolites. Fecal pellets (20 to 40) from each cage were collected 3 h after mice were placed into clean cages. Fecal pellets were collected at 4 time points during the study (weeks –1, 1, 6, and 12). Because fecal corticosterone metabolite levels fluctuate depending on the time of day, all samples were collected at 1100 (± 1 h). All samples were kept frozen in a –80 °C. At the end of the study, all samples were shipped to Charles River (Wilmington, MA) to be processed for analysis (catalog no. ab108821, Corticosterone ELISA Kit, Abcam, Cambridge, MA) according to the supplier’s protocols. Laboratory technicians who processed the samples and ran the assay were blind to all treatments.

Clinical pathology and immunophenotyping. At the termination of the study (24 to 48 h after the last dose), blood was collected from the abdominal aorta of isoflurane-anesthetized mice, processed, and analyzed (Figure 1). Due to the mouse’s limited blood volume, an entire biochemistry panel could not be run. Hematology, immunophenotyping, and creatinine were prioritized in light of predicted alteration due to our treatments. Given the known effects of cyclophosphamide on the kidneys and immune system,^{24,31} creatinine analysis, complete hematology, and immunophenotyping were selected. Blood was transferred to appropriate tubes as follows: 0.7 mL in serum separator tubes for clinical chemistry and 0.6 mL in K₃EDTA for hematology and immunophenotyping. The clinical chemistry samples were mixed gently and allowed to clot at room temperature for at least 30 min before being centrifuged for approximately 10 min in a refrigerated centrifuge (set to maintain 4 °C) at 1470 × g. Serum was then processed through an automated chemistry analyzer (Hitachi Modular P800 Analyzer, Roche Diagnostics, Indianapolis, IN) for creatinine levels. From the EDTA tube, a target volume of 0.3 mL of whole blood was sent for complete hematology analysis (Figure 1; ADVIA 120 Hematology System, Siemens, Munich, Germany). The remainder of the EDTA sample was mixed gently and kept under ambient condition until immunophenotyping analysis, in which the cellular antigens CD19 and CD3e and the B and T cell lymphocytes populations were quantified by using specific antibodies against the marker antigens. The technicians who ran the samples were provided with cage numbers only as identifiers, and no other treatment information was supplied.

Hematology parameters	Immunophenotyping parameters
RBC count	Markers:
Hgb concentration	CD3 ⁺ CD45RA ⁺
Hct	CD3 ⁺
MCV	Cell populations:
RBC distribution width	B lymphocytes
MCHC	T lymphocytes
MCH	
Platelet count	
Mean platelet volume	
Reticulocyte count (absolute)	
WBC count (total and absolute differential)	
Microscopic cell morphology	
Neutrophil count (absolute)	
Lymphocyte count (absolute)	
Monocyte count (absolute)	
Eosinophil count (absolute)	
Basophil count (absolute)	
Large unstained cell count	

Figure 1. Blood parameters evaluated at termination of the study.

All hematology and clinical chemistry results were interpreted by a board-certified veterinary clinical pathologist (AP).

Necropsy and histopathology. The cage order of euthanasia and sampling rotated through all treatment combinations, including controls, so that any potential diurnal variation would be distributed evenly across all treatments. After blood collection, mice were euthanized by exsanguination and diaphragm severing. Complete necropsy included evaluation of the carcass and musculoskeletal system; all external surfaces and orifices; cranial cavity and external surfaces of the brain; and thoracic, abdominal, and pelvic cavities with their associated organs and tissues. Brain, adrenal glands, thyroid gland, kidney, spleen, and thymus were weighed. Paired organs were weighed together. Organ weight:body weight ratios (using the terminal body weight) and organ weight:brain weight ratios were calculated. Because cyclophosphamide is an immunosuppressant that can cause kidney toxicity in mice at the dose level used in the current study, we collected various lymphoid organs, including one mandibular lymph node, spleen, thymus, adrenal glands, kidneys, and other organs with gross lesions and preserved the samples in 10% neutral buffered formalin. All collected tissues were embedded in paraffin, sectioned, mounted on glass slides, and stained with hematoxylin and eosin. Histopathology was performed by a board-certified veterinary pathologist (ED). Necropsy technicians, although not blind to nesting material in the cage, were blind to the nature of the study as well as the drug treatment.

Statistical analysis. All data collected were analyzed in repeated-measures mixed general linear models (JMP 11, SAS Institute, Cary, NC). Factorials are specifically designed to maximize power and reduce sample size because they incorporate, account for, and eliminate unwanted variance. The use of factorial and repeated measures design is commonly used as a means of reduction and refinement. This approach allows us to test for general effects of each variable and to look for interactive effects with greater power than nonfactorial analyses.²⁰ In addition, this design allowed us to test our treatments as we controlled for sex, bodyweights, and repeated measures per cage. The experimental treatment was applied to the cage of mice; therefore our experimental unit was the data at the cage level. When multiple measures were derived from mice in the same cage (for example, body weight), they were processed to a mean to assess the overall effect of treatment. A full-factorial analysis of nesting treatment, cyclophosphamide treatment, sex, and week was blocked by cage, nested within nesting treatment,

cyclophosphamide treatment, and sex. This model was used in all analyses unless otherwise stated. Higher-order interactions were dropped when nonsignificant. Fecal corticosterone metabolite data were analyzed as differences from baseline value (baseline – time point) to determine the overall change from levels before the start of the study. Due to lost data for food consumption (that is, spillage), cages were excluded from the analysis when 7 or more data points were missing from the total 13. This criterion included 1 cage from group 1, 2 from group 2, and 2 from group 4. Because the average body weights of cyclophosphamide-treated mice were significantly lower at the end of the study as compared with the saline-treated mice, organ-to-brain weight ratio was analyzed. Data that provided a yes or no outcome, such as pathologic results, were analyzed by a binary logistic regression to test similar parameters as listed for the generalized linear model analysis.

Our design covered 4 replicates ($n = 4$ per combination), which is 2 more than the minimum for a factorial design to account for potential animal loss (96 mice per 36 cages). The assumptions of generalized linear models (normality of error, homogeneity of variance, and linearity) were confirmed posthoc, and appropriate transformations were made to meet these assumptions.²⁰ Square-root transformation was performed for absolute T lymphocytes and absolute B lymphocytes. Adrenal:brain weight and thyroid:brain weight were log-transformed. Significant effects were analyzed posthoc by using Tukey tests or Bonferroni-corrected planned test slices. All values are given as least-squares means and standard errors. To determine whether our treatments affected the number of mice observed in or out of the nest during clinical observations we used a generalized linear model with Poisson distribution. A full-factorial analysis of nesting treatment, cyclophosphamide treatment, sex, and week was blocked by cage, nested within nesting treatment, cyclophosphamide treatment, and sex. A P level less than 0.05 was used to define significance for all tests. In several cases, the pathologists (ED and AP) considered that statistically significant differences were biologically irrelevant, as noted in the Results section.

Results

Analysis details are summarized in Table 1 for the following reported results. Posthoc tests are reported in full detail.

Clinical observations. Clinical observations of mice differed significantly depending on whether they were treated with cyclophosphamide and whether nesting material was present in the cage ($P < 0.001$; Figure 2). Piloerection, whiskers missing, and clear teeth occurred more often in cyclophosphamide-treated groups as compared with saline-treated groups ($P < 0.05$). Nested cyclophosphamide-treated mice had a lower ($P < 0.05$) incidence of erect fur than other groups. Nested saline-treated mice had significantly fewer missing whiskers as compared with nonnested saline-treated mice ($P < 0.05$). There was no effect of either of treatment on whether mice were recorded as in or out of the nest (nesting treatment, $P = 0.06$; cyclophosphamide treatment, $P = 0.14$).

As expected, the only difference in weight at baseline was between males and females ($P < 0.001$), with female mice being lighter. When compared over the course of the study, nesting treatment, cyclophosphamide treatment, and sex had significant effects on average body weight ($P < 0.001$; Figure 3). The average weight of male mice was highest in nested saline-treated mice and lowest in the nested cyclophosphamide-treated mice ($P < 0.05$). No intergroup differences occurred among female mice.

Food consumption was affected by nesting material and cyclophosphamide treatment ($P < 0.001$). In cyclophosphamide-treated mice, nonnested mice ate more than nested mice ($P < 0.05$). No differences were found between nesting treatments in saline-treated groups ($P > 0.05$). In addition, nesting material differentially affected food consumption between sexes ($P < 0.001$), such that nonnested female mice ate more than those with nesting material ($P < 0.05$). No differences in food consumption according to nesting treatment occurred in male mice ($P > 0.05$). Furthermore, food consumption differed between nonnested and nested cages over the 13-wk study period ($P = 0.027$). That is, nonnested mice ate more as the study progressed ($F_{1,259} = 13.9$; $P < 0.001$), whereas food consumption in nested cages was stable over the 13-wk period ($F_{1,259} = 0.05$; $P = 0.82$). Cyclophosphamide treatment affected food consumption over the course of the study ($P < 0.001$), and cyclophosphamide-treated mice steadily increased the amount they ate over time ($F_{1,259} = 17.7$; $P < 0.001$), but saline-treated mice showed no change ($F_{1,259} = 0.11$; $P = 0.74$).

Nest scoring. Nest scoring differed significantly according to nesting treatment, sex, and drug treatment (cyclophosphamide or saline; $P = 0.032$; Figure 4). Nested cyclophosphamide-treated male mice had significantly lower nest scores than nested saline-treated males ($P < 0.05$). However, nest scores did not differ between nested females regardless of drug treatment. In addition, nest scores of nonnested mice did not differ between sexes or drug treatment groups. However, nest scores varied over time depending on whether nesting material was provided ($P < 0.001$). Specifically, average nest scores of nested mice decreased from a score of approximately 4.75 to 3.8 as the study progressed ($F_{1,376} = 132.32$; $P < 0.001$), but nest scores of nonnested mice did not change and remained approximately 2.5 throughout the course of the study ($F_{1,376} = 0.30$; $P = 0.58$).

Fecal corticosterone metabolites. Fecal corticosterone metabolites varied significantly due to interaction between nesting material and drug treatment ($P < 0.001$; Figure 5). Metabolite levels did not differ among saline-treated mice ($P > 0.05$), but nested cyclophosphamide-treated mice had significantly lower levels of corticosterone metabolites than nonnested mice ($P < 0.05$). In addition, nonnested saline-treated mice had lower levels than nonnested cyclophosphamide-treated mice ($P < 0.05$). The average corticosterone metabolite levels of all nested mice, regardless of drug treatment, did not differ from their baseline values ($P > 0.0125$). However, regardless of drug treatment, nonnested mice all showed significant increases above baseline ($P < 0.0125$). Furthermore, both sex and nesting material treatment altered differences from baseline fecal corticosterone values ($P < 0.001$). Female nested mice had significantly lower values than nonnested females ($P < 0.05$), but nesting treatment had no effect on fecal corticosterone metabolites in male mice ($P > 0.05$). The time point at which samples were collected altered levels depending on nesting treatment ($P = 0.039$; Figure 6). No differences between nesting treatments emerged during the first week of the study ($P > 0.05$). However, at weeks 6 and 12, changes from baseline metabolite levels were significantly lower in nested mice compared with nonnested mice ($P < 0.05$). Changes from baseline metabolite values occurred over the course of the study depending on whether mice were treated with cyclophosphamide or saline ($P = 0.039$). Test slices revealed that cyclophosphamide-treated mice had higher corticosterone values during the first week of the study ($\alpha/3$; $P = 0.005$), but no differences were found at week 6 or 12 ($\alpha/3$; $P > 0.017$).

Clinical pathology. Weekly intraperitoneal injections of cyclophosphamide induced various hematology changes (Table 2).

Table 1. Statistical analyses

Measure	Significant effect	Degrees of freedom		Test statistic ^a	P
		Numerator	Denominator		
Clinical observations	Cyclophosphamide×nest	8	3576	26.6	<0.001
In or out of nest ^a	Nest	1		3.59	0.06
	Cyclophosphamide	1		2.14	0.14
Average body weight, baseline	Sex	1	24	448.9	<0.001
Average body weight, by week	Cyclophosphamide×nest ×sex	1	383	37.4	<0.001
Food consumption	Cyclophosphamide×nest	1	259	11.3	<0.001
	Nest×sex	1	259	23.2	<0.001
	Nest×week	1	259	5	0.027
	Cyclophosphamide×week	1	259	9.3	<0.001
Nest score	Cyclophosphamide×nest ×sex	1	376	4.6	0.032
	Nest×week	1	376	60.0	<0.001
Fecal corticosterone metabolites	Cyclophosphamide×nest	1	44	33.2	<0.001
	Nest×sex	1	44	38.2	<0.001
	Nest×week	1	44	3.5	0.039
	Cyclophosphamide×week	1	44	3.5	0.039
Absolute T-cell count	Cyclophosphamide	1	24	114.5	<0.001
	Nest	1	24	0.09	0.77
Absolute B-cell count	Cyclophosphamide×sex	1	24	24.5	<0.001
	Nest	1	24	1.4	0.24
Thymus weight	Cyclophosphamide	1	24	145.1	<0.001
Spleen weight	Cyclophosphamide	1	24	30.6	<0.001
Adrenal gland weight	Nest×cyclophosphamide	1	24	6.1	0.02
	Sex	1	24	266.6	<0.001

^aLikelihood ratio χ^2 for generalized linear model analysis and F value for general linear model

^bGeneralized linear model; all other analyses used a general linear model

These changes included minimal to moderate decreases in RBC mass parameters accompanied by decreased absolute reticulocyte counts in both male and female mice and marked decreases in lymphocyte and eosinophil counts. No significant differences were observed between nested and nonnested mice.

Immunophenotyping. When compared with saline-treated groups, cyclophosphamide-treated mice had significantly fewer T lymphocytes ($P < 0.001$). Cyclophosphamide differently affected the absolute count of B lymphocytes between sexes ($P < 0.001$): saline-treated mice had the highest numbers of B lymphocytes, regardless of sex, whereas cyclophosphamide-treated female mice had higher absolute numbers of B cells than did cyclophosphamide-treated males ($P < 0.05$). The provision of nesting material had no effect on T ($P = 0.77$) or B ($P = 0.24$) lymphocyte counts.

Necropsy and histopathology. All mice survived the 13-wk experiment. At necropsy, a small thymus was observed in 14 of the 24 male mice and 12 of the 24 female mice treated with cyclophosphamide; there was an increase in incidence developing

a small thymus in nested cyclophosphamide-treated mice (17 of 24) when compared with nonnested cyclophosphamide-treated mice (9 of 24). Small or fractured teeth (lower and upper incisors) occurred in 5 of the 24 cyclophosphamide-treated female mice, in both nested and nonnested groups. Enlargement and pale discoloration of the kidneys were present in 19 of the 48 cyclophosphamide-treated mice, regardless of sex or the presence of nesting material.

In the thymus and spleen, significant decreases in mean weight (relative to brain weight) were observed in all cyclophosphamide-treated mice ($P < 0.001$ for both comparisons). Adrenal gland weight was significantly altered depending on nesting material and cyclophosphamide treatments ($P = 0.02$): nested cyclophosphamide-treated mice had heavier adrenal weights than saline-treated mice ($P > 0.05$). In addition, adrenal weight (relative to brain weight) was heavier in female mice than males ($P < 0.001$).

Histopathologic findings included lymphoid depletion in the thymus, spleen, and mandibular lymph nodes in both nested

Figure 2. Percentage (least-square mean $\pm$ SE) of weekly detailed examinations during which clinical signs were present. Different letters indicate significant ($P < 0.05$) differences within clinical sign.

Figure 3. Body weight averaged by cage throughout the experiment. Differences due to sex, drug treatment (S, saline; CY, cyclophosphamide), and nesting treatment are shown as least-square means $\pm$ SE. Different letters indicate significant ($P < 0.05$) differences within sex; bars indicate significant ($P < 0.05$) differences in body weight between sexes. No effect on body weight in female mice was observed.

and nonnested cyclophosphamide-treated mice, at incidences of 47 of 48, 44 of 48, and 46 of 47, respectively. This change was characterized by minimal to marked reduction of cortical thickness and loss of clear corticomedullary junction in the thymus, minimally to markedly reduced size or number of splenic lymphoid follicles, and minimally to moderately reduced size or number of cortical lymph node follicles. In the adrenal glands, cortical hypertrophy occurred in cyclophosphamide-treated male mice (17 of 24) in both nested and nonnested groups. This change consisted of a minimal increase in thickness of the zona fasciculata in the adrenal cortex, with enlarged adrenocortical cells. In the teeth (lower and upper incisors), odontoblastic degeneration occurred in 3 cyclophosphamide-treated female mice. This change was characterized by the presence of few, scattered necrotic odontoblasts, mainly located at the base of the tooth pulp (preodontoblasts), with secondary atrophy or loss of dentin and macroscopic correlates of small teeth and fractures. Both saline-treated groups demonstrated only incidental macroscopic and microscopic findings.

Figure 4. Average nest score throughout the experiment. Differences due to sex, drug treatment (S, saline; CY, cyclophosphamide), and nesting treatment are plotted as least-square means $\pm$ SE; different letters indicate significant ($P < 0.05$) differences within sex.

Figure 5. Differences in corticosterone fecal metabolites from baseline values before the first injection of saline or cyclophosphamide averaged across the experiment. Mice were provided with bedding plus 10 g of crinkle paper nesting material (Nest) or bedding only (Control). Data are plotted as least-square means $\pm$ SE; different letters indicate significant ($P < 0.05$) differences within drug treatment, and bars indicate significant ($P < 0.05$) differences between drug treatments. Asterisks indicate significant differences according to t tests (values different from 0 or baseline; α corrected for 4 comparisons).

Discussion

Our current data establish that the provision of nesting material during a 13-wk cyclophosphamide toxicology study reduces stress, as measured according to fecal corticosterone metabolites, but does not alter major clinical parameters in mice. No differences in hematology, clinical chemistry, or absolute lymphocyte counts were observed between nested and nonnested mice. Furthermore, the provision of nesting material had no effect on histopathology findings. However, nested cyclophosphamide-treated mice had significantly lower levels of fecal corticosterone metabolites than did nonnested cyclophosphamide-treated groups. In addition, nested groups showed a lower incidence of clinical signs associated with general malaise.

Overall clinical pathology, gross necropsy, and histopathology findings were unaffected by the presence or absence of nesting material. The decreased absolute lymphocyte T and B counts in cyclophosphamide-treated mice are pharmacologic effects of cyclophosphamide. Decreases in RBC mass parameters with decreased absolute reticulocyte counts and decreases in absolute lymphocyte and eosinophil counts are reported effects of

Figure 6. Differences in corticosterone fecal metabolites from baseline values over the 13-wk experiment. Data are plotted as least-square means $\pm$ SE; different letters indicate significant ($P < 0.05$) differences within drug treatment according to Tukey pairwise comparison.

cyclophosphamide also.^{1,9,24} In our mice, decreases in lymphocyte counts correlated with histologic findings of minimal to marked lymphoid depletion in the thymus, spleen, and lymph nodes with macroscopic correlates of small thymus size and decreased mean thymus and spleen weights. These observed pathologic changes in lymphoid organs are well characterized in the literature.¹ All of the clinical pathology and histopathology changes observed occurred in cyclophosphamide-treated mice, regardless of nesting treatment, demonstrating that nesting material does not mask the expected effects of cyclophosphamide.

Our study showed that corticosterone metabolites were substantially lessened in cyclophosphamide-treated mice given nesting material, indicating reduced stress. The increase in corticosterone metabolite levels from baseline in the nonnested saline-treated mice may be attributable to the stress associated with the weekly injections, cold stress, and the lack of retreat space.^{10,19,39,43} Even more intriguing, stress levels in the nested saline-treated mice were not significantly increased from their baseline values. This finding potentially indicates that these mice, with average values across the whole experiment, did not experience any more stress than they had been experiencing prior to the start of the experiment. Predictability and the ability to control of stressors are very influential regarding how great a negative effect an experience will exert physiologically.^{34,48} Therefore it is plausible that thermal stress and the stress associated with repeated injections may have been reduced by access to a retreat space and ability to stay warm inside the nest or that injection stress alone is insufficient to significantly increase stress levels from baseline. Nonnested cyclophosphamide-treated mice, as expected, had the greatest increase in corticosterone metabolite levels. However, the ability of the nest to reduce stress levels in cyclophosphamide-treated mice was far greater than we expected. The stress levels detected in nested cyclophosphamide-treated mice did not differ from prestudy baseline values, indicating a protective effect of nesting material. In addition, nested mice appeared to cope more rapidly to the stress associated with our current experiment than did nonnested mice, regardless of drug treatment. This conclusion is supported by the fact that nested mice had significantly lower corticosterone values at weeks 6 and 12 than nonnested mice and is consistent with another study in which environmentally enriched mice were less affected by stress.⁵

Erect fur—piloerection—is one of the first clinical signs observed in mice experiencing general malaise, such as during pain or sickness.³⁸ The significant decrease in the incidence of this clinical sign suggests that nesting material reduces the feeling of general malaise due to cyclophosphamide treatment. However, the effect of nesting material did not mask the expected clinical signs of cyclophosphamide, as shown by the similar incidences of missing whiskers and clear teeth between the 2 cyclophosphamide-treated groups. In addition, the incidence of missing whiskers was significantly lower in nested saline-treated mice compared with nonnested saline-treated mice. This finding potentially can be explained by increased barbering behavior in the saline-treated, nonnested mice. Mice without access to a nest are cold-stressed,^{13,18} and stress has been shown to increase the development of abnormal behaviors.⁴⁹ We interpret this finding to suggest that cold stress was alleviated by the provision of nesting material. This interpretation is consistent with previous work⁴ demonstrating that environmental enrichment reduces the likelihood of alopecia and barbering in mice.

As expected, the cyclophosphamide-treated mice showed more abnormal clinical signs than the saline-treated mice. Cyclophosphamide is an alkylating agent, which works by causing crosslinks in dividing DNA, inhibiting DNA replication and causing cell death in rapidly dividing cells. Hair follicles in the anagen phase contain rapidly dividing cells responsible for hair growth. In adult rats, only 10% of hair follicles are in the anagen phase, as compared with 90% in humans,³⁶ thus explaining why rats don't develop significant alopecia in association with this chemotherapeutic compared with humans, in whom alopecia is the most common side effect. Rat whiskers, however, are constantly growing.²⁶ Given that mice present physiologic characteristics similar to those of rats, this effect might explain the increased incidence of missing whiskers observations in cyclophosphamide-treated mice. In addition, cyclophosphamide treatment resulted in odontoblastic degeneration and secondary loss of dentin, which are well-characterized effects of cyclophosphamide,^{2,30} in our mice.

Nesting treatment did not significantly alter T or B lymphocytes populations in our mice, illustrating that nesting material does not alter important immunophenotyping effects of cyclophosphamide. However, researchers who do not provide thermal support to their mice may see alterations in adaptive immunity. Recent work has demonstrated that the standard laboratory temperatures affect the fundamental properties and functions of dendritic cells, which play a vital role in the generation of effective and long-term immune protection from cancer and other diseases.³³ In long-term studies, a stronger immune system might positively affect animal longevity. Survival rate in control animals is a critical factor in carcinogenicity studies, and improving longevity might affect the successful outcome of long-term studies. For example, previous work has shown that the alleviation of stress in mice by housing at thermoneutrality consequently reduces tumor formation, growth rate, and metastasis.³² Housing mice at thermoneutrality creates other husbandry problems, given that this practice is associated with an increase in aggression.²¹ A practical alternative is to provide nesting material, which has been shown to decrease aggression and relieve thermal stress.^{13,45} Further research is needed to evaluate the effect of the provision of nesting material in the context of standard laboratory temperatures on tumor growth.

Mice provided nesting material demonstrate a reduction in heat loss, which leads to a decrease in energetic needs reflected in decreased food consumption.¹³ In addition, cyclophosphamide

Table 2. Statistical analysis of clinical pathology parameters

	Nesting treatment				Drug treatment			
	No nest	Nest	F ratio	P	Saline	Cyclophosphamide	F ratio	P
RBC ($\times 10^6/\mu\text{L}$)	6.99 $\pm$ 0.08	7.09 $\pm$ 0.08	0.78	0.39	7.41 $\pm$ 0.11	6.67 $\pm$ 0.11	16.90	<0.001
Hgb (g/dL)	10.91 $\pm$ 0.16	11.01 $\pm$ 0.16	0.19	0.67	11.43 $\pm$ 0.21	10.48 $\pm$ 0.21	6.75	0.016
Hct (%)	34.62 $\pm$ 0.48	35.17 $\pm$ 0.48	0.66	0.43	36.24 $\pm$ 0.62	33.56 $\pm$ 0.62	6.45	0.018
MCV (fL)	49.59 $\pm$ 0.31	49.63 $\pm$ 0.31	0.0070	0.95	48.84 $\pm$ 0.40	50.38 $\pm$ 0.40	5.14	0.033
MCHC (g/dL)	31.48 $\pm$ 0.11	31.28 $\pm$ 0.11	1.68	0.21	31.54 $\pm$ 0.15	31.28 $\pm$ 0.15	1.78	0.1947
RBC distribution width (%)	14.37 $\pm$ 0.21	14.39 $\pm$ 0.21	0.0066	0.94	13.34 $\pm$ 0.28	15.43 $\pm$ 0.28	19.83	<0.001
Platelet count ($\times 10^3/\mu\text{L}$)	1248.91 $\pm$ 15.83	1232.76 $\pm$ 15.83	0.51	0.48	1122.90 $\pm$ 20.73	1358.77 $\pm$ 20.73	45.44	<0.001
Mean platelet volume (fL)	5.64 $\pm$ 0.12	5.62 $\pm$ 0.12	0.025	0.88	5.65 $\pm$ 0.16	5.61 $\pm$ 0.16	0.029	0.87
Reticulocytes ($\times 10^9/\text{L}$)	242.95 $\pm$ 13.26	243.50 $\pm$ 13.26	0.0009	0.98	357.17 $\pm$ 17.37	129.28 $\pm$ 17.37	60.45	<0.001
WBC ($\times 10^3/\mu\text{L}$) ^a	2.72 $\pm$ 0.0036	2.86 $\pm$ 0.0036	0.15	0.71	4.54 $\pm$ 0.0064	1.44 $\pm$ 0.0064	47.77	<0.001
Neutrophils ($\times 10^3/\mu\text{L}$)	0.63 $\pm$ 0.04	0.61 $\pm$ 0.04	0.16	0.69	0.52 $\pm$ 0.06	0.72 $\pm$ 0.06	4.67	0.041
Lymphocytes ($\times 10^3/\mu\text{L}$) ^a	1.81 $\pm$ 0.0036	1.93 $\pm$ 0.0036	0.24	0.63	3.81 $\pm$ 0.0061	0.62 $\pm$ 0.0061	77.25	<0.001
Monocytes ($\times 10^3/\mu\text{L}$)	0.06 $\pm$ 0.01	0.07 $\pm$ 0.01	0.34	0.57	0.09 $\pm$ 0.01	0.05 $\pm$ 0.01	8.46	0.0080
Eosinophils ($\times 10^3/\mu\text{L}$)	0.081 $\pm$ 0.0055	0.068 $\pm$ 0.0055	2.70	0.11	0.11 $\pm$ 0.0072	0.043 $\pm$ 0.0072	27.49	<0.001
Basophils ($\times 10^3/\mu\text{L}$)	0.0019 $\pm$ 0.00071	0.0026 $\pm$ 0.00071	0.45	0.51	0.0035 $\pm$ 0.0093	0.00097 $\pm$ 0.00093	2.65	0.12
Large unstained cells ($\times 10^3/\mu\text{L}$)	0.028 $\pm$ 0.003	0.028 $\pm$ 0.003	0.0056	0.94	0.036 $\pm$ 0.004	0.020 $\pm$ 0.004	6.45	0.018
Creatinine (mg/dL)	0.11 $\pm$ 0.019	0.11 $\pm$ 0.019	0.075	0.79	0.13 $\pm$ 0.025	0.095 $\pm$ 0.025	0.50	0.49

^aData were transformed to meet the assumptions of a general linear model. Therefore the data presented here have been back-transformed to represent actual values.

treatment is commonly associated with decreased appetite.²⁴ Therefore, we expected nested mice to either eat less or weigh more.¹³ However, our results failed to demonstrate this effect consistently: there was no difference in food consumption according to nesting treatment in saline-treated groups or drug treatment in male mice. In addition, body weight in female mice did not differ due to either nesting or drug treatment. However, as expected, cyclophosphamide-treated male mice weighed less than saline-treated males, indicating the better health of the saline-treated mice.

Among the saline-treated male mice, those with nests were the heaviest. Nonnested saline-treated males were likely thermally stressed and reallocated considerable energy to maintaining thermoregulation, thus explaining their lower mean body weight. Among cyclophosphamide-treated males, we expected the nested mice to have higher body weights; instead, the nonnested mice were heavier. Nest scores from cyclophosphamide-treated males were significantly lower than those from saline-treated males. Therefore, we believe that cyclophosphamide-treated males, experiencing general malaise, were not building optimal nests. Accordingly, we then expected body weight to be similar between the 2 nesting treatments, contrary to what in fact occurred. At this point, we cannot explain why nonnested cyclophosphamide-treated male mice were heavier. If behavior had been recorded, it might have shed light on the overall activity levels of these 2 groups. It is also interesting to note that the lack of difference due to nesting treatment on the weight of female mice. We expected a similar pattern as that in the saline-treated males. Previous literature indicates that even with 8 to 10 g of nesting material, female mice might still experience thermal stress.^{12,13} Therefore, perhaps the female mice in our experiment did not experience sufficient relief from thermal stress to alter their body weights.

In the current study, cyclophosphamide seems to have had a greater effect on nest building capacity in male mice than in female mice. Previous literature indicates that behavioral changes in sick animals are the expression of the reorganization

of highly motivated behaviors.³ Female mice potentially are more thermally challenged than are males^{12,13} and therefore, in the context of general malaise, retain their motivation to engage in nest building activities that minimize heat loss. This motivation may explain why cyclophosphamide treatment had no effect on nest building by female mice. As for male mice, which seem to be less thermally stressed than females at the same temperature,¹² the motivation to decrease thermal stress was not nearly as strong as general malaise; consequently, male mice engaged less in nest building than females, resulting in lower-scoring nests.

We conclude that nesting material had no negative effects on the outcomes of a 13-wk repeated-dose cyclophosphamide toxicology study. All cyclophosphamide-related changes occurred with similar incidence and severity in both nested and nonnested (control) groups. Furthermore, mice given nesting material showed decreased overall stress, as measured by corticosterone metabolites, and appeared to acclimate faster to cyclophosphamide-injection-related stress. In addition, the decreased incidence of clinical signs of general malaise suggest that the nested mice were more comfortable. Nesting material again was confirmed to be a biologically relevant enrichment that improved animal welfare in the absence of effects on study parameters. We therefore recommend the use of nesting material in cyclophosphamide toxicology studies and encourage toxicologists to consider using crinkle paper nesting material during toxicology testing of other compounds.

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