Environmental Health Perspectives
Vol. 96, pp. 199-202, 1991

Cage Allocation Designs for Rodent Carcinogenicity Experiments

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Cage allocation designs for rodent carcinogenicity experiments are discussed and presented with the goal of avoiding dosage group biases related to cage location. Considerations in selecting a cage design are first discussed in general terms. Specific designs are presented for use in experiments involving three, four, and five dose groups and with one, four, and five rodents per cage. Priorities for balancing treatment groups include horizontal position on shelf and shelf of rack, nearest neighbor balance, and male-female balance. It is proposed that these balance criteria be considered together with practical issues, such as the ability to accurately conform to a design and to determine a sensible and efficient design for each experiment.

Introduction

The validity and sensitivity of rodent carcinogenicity experiments for assessing the safety of food additives, drugs, cosmetics, and other substances depend a great deal on the experimental design. Some of the many issues that need to be considered and implemented before initiating an experiment are the number of dose groups, the choice of dose levels, the strain of mouse or rat, the number of animals per dose, the number of animals per cage, and the allocation of animals to dose groups. There are also numerous design issues that are implemented during and upon completion of the experiment, such as feeding schedules, monitoring of animals, time of interim or terminal sacrifices, and pathology review. For detailed discussions of many of these issues, see Fox et al. (*1*), Gart et al. (*2*), Grice et al. (*3*), Krump (*4*), Portier and Hoel (*5–7*), Greenman et al. (*8*), Haseman (*9*), and Bickis and Krewski (*10*).

This paper considers a design issue other than those mentioned, namely, the allocation of dose groups to cages. In most laboratories, animal cages are arranged on racks having four to six shelves and holding from five to eight cages per shelf. Usually each rack is aligned with another rack; therefore, one can consider the pair as a single rack having a front and a back section. When racks are paired in this way, it also is common to place male animals in one section, for example, the front, and females in the other. The decision of where on the rack to locate the different dose groups is important because there can be environmental differences that influence a rodent's longevity or risk of developing a tumor. For example, cages in different locations on racks experience different temperature, humidity, and lighting conditions based on height, proximity to ventilation devices, lights, or fans, and perhaps even noise. If dose groups are assigned to cages in a systematic way, these environmental effects can bias the statistical analyses used to assess whether dose has an effect on tumor production. For example, if each shelf on the rack corresponds to a single dose group and there are altitude effects, then there will be a systematic bias that could cause a spurious association between dose and tumor rates or mask a real association. Such effects have been noted in several experiments (*8,11,12*).

One approach to overcoming such a bias is by controlling for shelf location in the analysis of the data. In an examination of data on red dye 40 (*11*), this would not be possible because shelf and dose are completely confounded. Even if there were only partial confounding, this is not a very desirable solution because controlling cage position requires that the appropriate model be selected. Lagakos and Mosteller (*11*) state that good designs for these experiments should have included balance in the layout of the cages, i.e., "to arrange cages in a way that 'balances' treatment groups with respect to rows, columns, positions and racks." They "prefer a balanced design to completely random allocation because it ensures that factors of interest will not be confounded, it leads to slightly more sensitive tests and it is easier to implement " (*11*).

A simpler, more foolproof, and more efficient solution than the above approach is to avoid the problem through design; that is, to prevent systematic bias from occurring. One way of achieving this is to use a completely random allocation of dose groups to cages. That is, once cages have been loaded onto racks, dose groups are assigned to cages in a completely random manner. Such an approach has a number of merits, the main one being that completely random allocation tends to prevent systematic biases. However, there are three potential difficulties: *a*) even though a fully randomized design will be balanced on average, imbalances can still occur; *b*) the process of randomly assigning doses to cages can be somewhat time consuming; and *c*) it might be more complicated for the laboratory technician who feeds the animals to keep track of the dose groups than in a

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systematic design, say where each rack row corresponded to a different dose, thus increasing the risk that animals are given the incorrect dose of the test compound.

The goal of this paper is to discuss the use of designs that force certain types of balance and, in so doing, avoid the first two of these problems. In the next section, specific designs for experiments involving three, four, and five dose groups are presented, along with the discussion of some related points. These designs are not an exhaustive selection but serve to illustrate what can be done in practice. Once the design has been determined, the assignment of dose levels, etc., should be done at random. For methods of performing such randomizations, see, for example, Cox (*13*).

Proposed Designs

Design Priorities

Experiments consisting of 50 animals per sex per dose group, which is customary in experiments conducted by the National Toxicology Program and in numerous privately conducted experiments, are considered. It is also assumed that any balancing by weight, litter, etc., has already taken place (*8*), and all that remains is the arrangements of cages onto racks and the allocation of cages to dose groups. A helpful guide on how to choose factors in an experiment is given in Cox (*13*).

In selecting designs for balancing dose groups by cage position, our priorities are *a*) Latin square balance: dose groups are balanced with respect to the "rows" (i.e., horizontal position) and "columns" (i.e., vertical position) within a rack, with each dose group appearing the same number of times in each row and column; *b*) nearest neighbor balance: cages to the north, south, east, west of each cage are balanced by dose group (*14*); and *c*) front–back balance: dose groups are balanced with respect to pairs of cages in the front and back of racks, referred to as orthogonality. It is usually not possible to achieve perfect balance according to these criteria; therefore, one attempts to use designs that come as close as possible. Similarly, given the number of animals per cage, it is not always possible to achieve exactly 50 animals per dose and sex.

Designs that satisfy the first and third of these criteria are referred to in the literature as orthogonal Latin squares or Graeco-Latin squares. For the analysis of such designs, see, for example, Box et al. (*15*) and Davies (*16*). Because one has balanced as much as possible and randomized where it is not possible to balance, the analysis of the designs satisfying the criteria will be as precise as possible.

Designs for Three Dose Groups

Let A, B, and C denote the three dose groups. Then 150 male and 150 female animals are needed for the experiment. Consider first the situation with four animals per cage; then 38 cages for each sex are needed, two cages having three animals. Figure 1 gives a design for this situation with eight cages per shelf. If shelves cannot hold this many cages, two racks can be used. The 18 interior cages in each rack are balanced in the rows and columns and for nearest neighbors, with each cage having two cages of each of the other two dose groups as nearest neighbors. Also, there is front–back balance, i.e., orthogonality between the front and back racks, and the Latin square property holds. The boundary cages of each rack cannot be completely balanced under the three criteria in the previous section, but are balanced as nearly as possible. The design is implemented by randomly assigning the letters A, B, and C to the three dose groups and then placing animals in their appropriate cages.

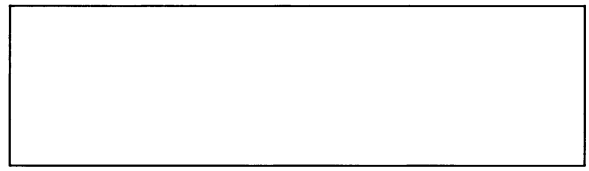

FIGURE 1. Cage layout for three dose groups, denoted A, B, and C with four animals per cage. Two letters within each cage denote dose groups for front and back of rack, respectively.

If five animals are housed in each cage, 30 cages per sex are necessary. Figure 2 gives a design for this situation. The middle three rows of cages are balanced for the three criteria of the previous section. With one animal per cage, 150 cages are needed for each sex. The design given in Figure 2 can be repeated five times for this.

Designs for Four Dose Groups

Let A, B, C, and D denote the four dose groups. Then 200 animals per sex are necessary for the experiment. Consider first the situation with four animals per cage; then 50 cages are needed per sex. An example of a design for this situation is given in Lagakos and Mosteller (*11*). Their design is balanced for rows and columns, orthogonality between the racks, has the Latin square property, and is also such that each of the four 2×2 quadrants in each 4×4 Latin suare contains all four dose groups. Figure 3 gives an alternative design based on the criteria discussed earlier. In this design, 48 cages are used for each sex, which results in the use of 192 animals. The design is balanced for the criteria except for orthogonality and the Latin property in each column. Each cage has as its nearest neighbors all four dose groups.

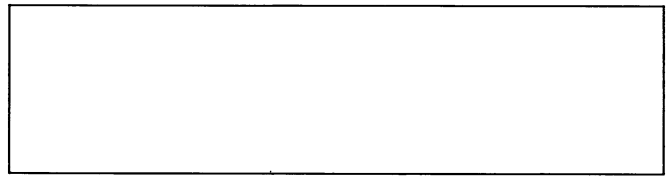

FIGURE 2. Cage layout for three dose groups, denoted A, B, and C with five animals per cage. Two letters within each cage denote dose groups for front and back of rack, respectively.

FIGURE 3. Cage layout for four dose groups, denoted A, B, C, and D with four animals per cage. Two letters within each cage denote dose groups for front and back of rack, respectively.

D,B	A,A	B,D	C,C	D,B	A,A
D,D	C,A	B,B	A,C	D,D	C,A
B,D	C,C	D,B	A,A	B,D	C,C
B,B	A,C	D,D	C,A	B,B	A,C

B,D	C,C	D,B	A,A		
B,B	A,C	D,D	C,A		
D,B	A,A	B,D	C,C		
D,D	C,A	B,B	A,C		

FIGURE 4. Cage layout for four dose groups, denoted, A, B, C, and D with five animals per cage. Two letters within each cage denote dose groups for front and back of rack, respectively.

With five animals per cage and four dose groups, 40 cages are needed for each sex. Figure 4 gives a design for this situation. The design is balanced inside the boundary, i.e., the inner 32 cages of each satisfy all criteria except for orthogonality and the Latin property in the columns.

Designs for Five Dose Groups

Let A, B, C, D, and E denote the five dose groups. Then 250 male and 250 female animals are needed for the experiment. With four animals per cage, approximately 62 cages are needed. Figure 5 gives a design for this situation with 60 cages for each sex. The design is balanced inside the boundary for the three criteria discussed earlier. The cages on the boundary are not balanced for nearest neighbor nor orthogonality. Inside the boundary each cage has each of the four other dose groups as its nearest neighbor.

With five animals per cage, 50 cages are necessary. Such a design is given by the cages inside the boundary of the design in Figure 5. For one animal per cage, 250 cages are necessary, and a design is given by five replicates of the design with five animals per cage.

Discussion

Two alternatives to the proposed designs are completely randomized designs and partially randomized designs that control for fewer factors. In most applications, it is our view that the designs proposed in this paper are preferable to a completely randomized design. The main reason for this is logistic simplicity: to use one of the designs given in this paper, one only needs to allocate the numbers $1,2,\ldots,k$ to the k dose groups. In contrast, a completely randomized design essentially requires $100k$ random allocations. The proposed designs also have the advantage of ensuring balance of dose groups with respect to shelf, location on shelf, and nearest neighbor, whereas the completely randomized design leaves this to chance.

The choice between the designs proposed in this paper and simpler balanced designs is less clear. One such design balances only with respect to shelf by having the same dose group in each column of a rack (*8,10*). This type of design will prevent biases from altitude effects, but does not guard against horizontal environmental effects. However, if there are concerns about the ability of laboratory technicians to give the appropriate doses to cages in feeding experiments, or if vertical spilling of feed is a real possibility, this design may be preferable. One must weigh the trade-offs of unexpected errors in food distribution versus unexpected environmental effects. In general, if the use of a particular design is likely to lead to serious error in the delivery of the assigned doses, then it would be prudent to use a cage allocation design that would minimize or avoid this problem.

E,C	A,A	B,D	C,B	D,E	E,C
C,D	D,B	E,E	A,C	B,A	C,D
A,E	B,C	C,A	D,D	E,B	A,E
D,A	E,D	A,B	B,E	C,C	D,A
B,B	C,E	D,C	E,A	A,D	B,B

A,A	B,D	C,B	D,E	E,C	A,A
D,B	E,E	A,C	B,A	C,D	D,B
B,C	C,C	D,D	E,B	A,E	B,C
E,D	A,B	B,E	C,C	D,A	E,D
C,E	D,C	E,A	A,D	B,B	C,E

FIGURE 5. Cage layout for five dose groups, denoted A, B, C, D, and E, with four animals per cage. Two letters within each cage denote dose groups for front and back, respectively.

When a restricted randomization is used in the design of an experiment, failure to account for this in the analysis can lead to conservatism in statistical tests for a dose effect on tumor rates, but in most situations this will be slight and thus of no concern (*17,18*). Alternatively, cage location can be controlled for in the analysis by regarding shelf height or location as explanatory variables and by using the regression model generalizations of the standard statistical methods of analysis (*19,20*).

Finally, note that the proposed designs can be used in conjunction with any type of scheme for allocating animals to dose groups. For example, if animals are assigned to dose groups in a completely or restricted randomized way to control for possible effects of weight, litter, etc., this allocation can precede the allocation of dose groups to cages.

Nothing is sacred about the design sizes that have been used here. They have been chosen only because of their similarity to the sizes used in actual experiments. They have been used as illustrations to show what is available in practice.

This research was supported by grant CA33041 from by the National Cancer Institute and by the Natural Sciences and Engineering Research Council of Canada. We are grateful to the reviewers for their useful comments and to Karen Abbett for her help in preparing this manuscript.

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