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Produced by the NASA Center for Aerospace Information (CASI)
ANNUAL REPORT

September 4, 1974 to September 4, 1975

NATIONAL AERONAUTIC AND SPACE ADMINISTRATION

CONTRACT NAS 9-14341

Effects of Weightlessness on Tissue Proliferation

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Our studies were induced by the observation that during American space flights, the astronauts have consistently sustained a significant reduction in red cell mass (astronaut anemia). Existing evidence, largely derived from the data on the recovery of red cell mass, suggested that marrow stromal dysfunction might have contributed to this "astronaut anemia". Thus, our studies during the past year have centered upon the repair of marrow stroma after mechanical injury. These studies are being carried out in order to obtain baseline data for an anticipated space experiment, the outline of which we have proposed for ground-based simulation (see below). The objective of the space experiment is to study the effect of weightlessness on marrow stroma as well as other proliferating cell systems.

During the past year the following specific areas have been investigated with the following results:

1. **Morphometric Studies on the Marrow Stromal Repair after Mechanical Injury:** Hemopoietic function of the bone marrow depends, on the one hand, upon proliferating hemopoietic cells and, on the other hand, upon a unique adventitial microstructure that supports hemopoiesis. When hemopoietic depression results from transient insult to proliferating hemopoietic cells, the recovery after the cessation of insult is relatively rapid. On the other hand, the marrow adventitia has a relatively slower turnover rate and its recovery after a transient insult is slow. Thus, when the hemopoietic depression results from injury to the marrow's supporting adventitia, the hemopoietic recovery is delayed. The delayed recovery of red cell mass in the post-exposure period may therefore indicate injury to the marrow's supporting structure.
We have used two experimental models for studying marrow stromal repair after injury. The repair process is similar in both models and has been shown to originate from marrow's stromal element and independent of marrow's hemopoietic cells. The first model consists of disruption of a marrow core in rat femur by inserting and withdrawing a slender trocar for the whole length of femoral cavity. Under ether anesthesia, the knee joint is opened and a hole is drilled in the articular surface of the femur until the marrow cavity is reached. The operation is simple and rapid. The animals are sacrificed at daily intervals or every 2-3 days, the bone is removed, cleaned from the surrounding soft tissues, in 10% buffered formalin and then decalcified. The bones are, then, bisected longitudinally and the open faces are sectioned after paraffin embedding. Longitudinal section of the whole bone is then mounted on glass slides and stained. Multiple photographs are obtained and their prints are mounted together in order to reconstruct the bone.

The sequence of repair process is similar to the sequence of marrow regeneration after autotransplantation of marrow fragments to ectopic sites and comprises nine well-defined steps:

1. Hemorrhage
2. Fibroblastic proliferation
3. Osteoblastic differentiation
4. Osteoid bone formation
5. Formation of primordial marrow cavity
6. Formation of marrow sinusoidal system
7. Appearance of hemopoietic cell foci
8. Hemopoietic cell production and expansion
9. Bone resorption

Each of these processes was quantified by cutting the reconstructed photographs of the whole bone mount and weighing the pieces of paper. The percentages of the surface areas for each point during the repair process was plotted against time and the results are shown in the appendix I. Each point represents the mean and standard deviations for 20 animals. More than 300 animals were used in these studies.

The second model is ablation of femoral cavity by a polyethylene tube, using a surgical technique similar to that described for the first model. In this model a rim of marrow is left in the proximal end of femur. Repair of marrow originates from this rim and gradually moves down to replace the ablated portion of the marrow. All nine steps, as described above, are seen. The regenerative boundary is well-defined and consists of proliferating fibroblastic tissue. By measuring the distance this regenerative boundary moves along the length of bone marrow cavity one may then give a quantitative dimension to the process of stromal repair. Because the first model was more predictable and consistent in the results, we have abandoned this second model.

2. Radioautography and cell kinetic study of stromal repair: These studies have just begun. The experimental designs are similar to what is described in the previous section. Animals are, however, given a pulse of $^3$H-Thymidine 24 hours before study. Radioautographs are being prepared according to standard methods and proliferating population of cells are studied. We shall extend these studies to examine the fate of the radioactive label in order to gain some information with
regard to the differentiation of various cell population during the repair of marrow stroma. We shall particularly focus on the bone formation and resorption in this model because, in anticipation of performing similar studies in space, the effects of weightlessness on bone formation and resorption in this model because, in anticipation of performing similar studies in space, the effects of weightlessness on bone formation and resorption and calcium metabolism would be of utmost interest. Baseline data, therefore, may be needed.

3. Effects of x-irradiation on repair of marrow stroma: These studies have just begun. The tibia is used. The tibial marrow is damaged by a method similar to what is described. At various times after operation, the tibia is subjected to local irradiation while the rest of the animal's body is shielded. For this purpose we have constructed special lead cage which allows shielding of animal's body excepting one tibia. The marrow in the opposite tibia is also subjected to mechanical damage but not to x-irradiation. It will be used as internal control. Different dose of radiation are being attempted to determine the radiosensitivity of every step in the repair process.

4. Preparations for ground-based simulations: We have made a preliminary proposal for a ground-based simulation study of stromal repair in bone marrow after mechanical injury. We are in the process of making a final protocol for this study to be carried out in L.B. Johnson Space Center by the end of this year. These simulation studies make it possible to evaluate the feasibility of designing an experiment, to be carried out during a future space expedition. We also plan to carry out a similar experiment in a ground-based setting where the G forces are increased. This can be done in a chamber where experimental animals are subjected to centrifugal forces. The information we
may gain from these studies permit the extrapolation of the results obtained in space (weightless state) to determine the effect of gravity on repair of bone marrow repair, calcium and bone metabolism and cell and tissue proliferation. We hope this would be a worthwhile contribution to NASA Life Science Program in space. Our preliminary proposal, phase II of these studies, is included as Appendix 2.

5. Design of small animal cage for space experiments: One of the problems that Life Science experimentation in space will have to face, is to redesign the usual experimental devices, equipments and instruments in such a way as to be applicable to experimentation in weightless state. The Russian experience aboard the Cosmos 605 Biosatellite (Aviation, Space and Environmental Medicine 46:319-321, 1975) suggest to us that some of the experimental rats died of starvation because in the weightless state they could not feed themselves. Two factors could have influenced this situation. Firstly, in the weightless state, the animals float in the midst of the cage, being unable to reach the source of food and water. Secondly, that the animal on ground, develop a ceiling-floor concept which is immaterial in space. To overcome these problems, we have designed a cage that prevents these 2 particular problems in future space experiments in which small animals are to be used. The outlay of this design with description is included in Appendix 3.

6. Effects of Hyperoxia: In one original proposal we had indicated our intention to study the effect of hyperoxia on the repair of marrow stroma. However, because available evidence indicates that hyperoxia is not responsible for the decrement of red cell mass seen during and after space expeditions. Following the Gemini and Apollo programs, hyperoxia was considered to be the main factor in the decrement of red cell mass seen in those flights. Hyperoxia can produce loss of red cell mass through erythropoietin production or hemolysis or both. However, the experience with Skylab flights, where oxygen concentration was less
than 100%, indicates that pure oxygen is not the major cause of red cell mass deficit. As a result, we have abandoned our efforts to study the effect of hyperoxia on the repair of marrow stroma.
APPENDIX 1

The Results of Morphometric Studies of Bone Marrow

-Repair After Mechanical Injury
Phase II
SKYLAB MISSION SIMULATION
Proposal for an Experiment

Objectives:

1. To establish the procedures for an orbital experiment in which samples of bone and bone marrow are obtained, by simple surgical technique, fixed chemically and stored for post-flight analysis.

2. To establish the training procedures for astronaut-type personnel to perform the surgical and tissue fixation procedures.

3. To develop, adapt and design laboratory instruments and tools necessary for carrying small animals to spacclabs, performing simple surgical procedures, fixing and storing tissues for post-flight examination.

4. To design an experiment in which the chronology of marrow repair after stromal injury is determined outside the gravitational field.

Experimental Design:

The experiment is designed for 12 small 100-150 gram Wistar rats which will be obtained from Simonsen Laboratory in California (where the animals for baseline studies are obtained from). The animals will be sent to Johnson Space Center. The animals will be divided into 2 groups of 6 and each group will be placed in separate cages, the design of which has been proposed in our previous reports. In addition 40 animals (20 in each group) will be treated in a similar fashion for ground-based control. Although the two groups of animals will be placed in separate cages to make their differentiation easy, as an additional means of differentiation the animals
in group 1 will be marked on the right ear and the animals in group 2 will be marked on the left ear.

The animal cages will be placed in the spacecraft under normal temperature. It is preferable that within the modular space, provided for animal housing, the rats be shaded in cycles of 8 hours in every 24 hours because rats are night feeders and the absence of these cycles may interfere with their feeding.

The animals will be prepared prior to launching in Johnson Space Center by an investigator from our laboratory. The operation shall consist of marrow disruption by an established surgical technique. The animals in group 1 will be killed and the femur will be removed 14 days after operation when, according to our morphometric studies, the hemopoietic proliferation reaches a peak. The animals in group 2 will be killed and the femurs will be removed for the study 5 days after operation when osteoid bone formation reaches a peak. Accordingly the following schedule is designed for this experiment (the mission is considered to be 7-days long):

<table>
<thead>
<tr>
<th>PRE-FLIGHT</th>
<th>FLIGHT</th>
<th>POST-FLIGHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Operate Group 1</td>
<td>Operate Group 2</td>
<td>Removal of tissue Group 2 Group 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Change Fixative Preliminary Report</td>
</tr>
<tr>
<td>-7</td>
<td>-1</td>
<td>0 1 2 3 4 5 6 7 1P 7P</td>
</tr>
</tbody>
</table>
Animals in group 1 will have surgery 7 days before the flight and the tissue will be removed on day 6 of flight (14 days post-surgery) when the hemopoietic cell proliferation reaches a peak. The animals in this group will be subjected to weightlessness during the last part of the repair process in the bone marrow, and any difference with the control group will reflect the sensitivity of these last sequences of repair process to weightless state.

Animals in group 2 will have surgery 1 day prior to launching and the tissue will be removed on day 4 of flight (5 days post-surgery) when osteoid bone formation reaches a peak. The animals in this group will be subjected to weightlessness during the first part of the repair process in the bone marrow and any difference with control groups will reflect the sensitivity of earlier sequences of repair process to weightlessness.

The tissue obtained during flight shall be received by one of us on the day of launching and it will be transferred to buffer from fixative and will be processed for histological examination, the preliminary result of which will be available one week after landing.

Equipment Required for Spacecraft:

1. Fixative vials, 12 in number (one for each animal) clearly marked or colored for group 1 and 2. The design of vials will be submitted later. Zenker's solution appears to be the most suitable fixative for the use in space as it is not aldehyde-based and as such, it is not volatile. The fixative vials should be put in a plastic security container which will be attached to the animal cage.
2. Two sets of surgical instruments, one for each group of animals. Every set contains (a) one knife with blade, (b) a small pair of one point sharp scissors, (c) a Kelly clamp, (d) small bone cutter.

3. The animals should be killed by CO$_2$ or vacuum (whichever is most technically and conveniently available in space craft). The cage should be contained in a heavy-duty, air-tight plastic bag with suitable vent outlet for attaching the vacuum or CO$_2$ source.

Astronaut Training and Flight Cookbook:

Four sessions of 3 hours each is considered sufficient for training of personnel who will kill the animals, surgically remove the specimen and insert it into fixative vial. The process will consist of the following preliminary steps:

1. Insert the cage in plastic bag.
2. Close the bag air-tight.
3. Evacuate the bag with vacuum source.
4. Wait at least 15 minutes or until rats appear dead.
5. Open the bag and remove it from the cage.
6. Remove animals one by one.
7. Use the bag as working surface.
8. Take scissors, using sharp point to penetrate the skin in area of hip--cut the skin to expose the joint.
9. Take bone cutter and remove the leg by cutting the hip.
10. Using the scissor cut away as much soft tissue as possible down to the knee joint.
11. Using the bone cutter, cut the knee joint.
12. Open the fixative vial's cap.
13. Place the bone into the vial.
14. Replace cap tightly.

15. Place the vial into security container.

The tissue samples need not be refrigerated. It is estimated that all the operations will last 2 hours for each group such that a total of 4 hours of flight-time will be needed for the experiment (2 hours on day 4 and 2 hours on day 6).
DESCRIPTION

Small Animal Cage for the Use in Space Bioexperiments

The cage (page 1) is 20 x 10 x 6 inches and except for the height of 6 in., is comparable to the cages that are routinely used in most laboratories. The height of 6 in. is so designed as to be slightly less than the arm span of an average rat. This permits the animal to hold onto the top and bottom faces of the cage preventing the animal's floating. Four animals can be housed in one cage, and 3 cages may form a rack (page 3). Because rats are night feeders, the rack should be shaded for at least 8 of 24 hours.

There is a rod, one inch in diameter, going from corner to corner (p. 1). The cage can rotate on this axis. While on the ground, the animals will be placed in the rotating cage for a period of several days in order to condition them to lose the floor-ceiling concept.

The top and bottom face are made of a galvanized net of 1/4 in. (p2). The long side surfaces are solid surfaces (p2) and the front surface has a circular arm hole, 4 in. in diameter to remove and replace the animals. The front surface also is site of water and food supply. The intake may be designed as a paste-like material to replace both water and food intake. (We have developed such a paste in our laboratory).

The small side faces (p2) are made of tubular bars, 1/4 in. in diameter, spaced at 1/2 in. Air flow in the long axis would keep the cage clean of excretion.

Within the dimension of the modular space provided for animal housing in the shuttle, the racks will be fitted together with the air pump and air filter to provide the laminar air flow.
Galvanized net surfaces
(Top and Bottom faces)

Rod (one inch diameter)
(Corner to corner)

Tubular bar surfaces
(Small side faces)

Food

Arm Hole

Water

Solid Surfaces
(Long Sides Faces)
Top and Bottom Faces

Galvanized Net Surface

Long Sides Faces

Solid Surface

Small Sides Faces

Tubular Bar Surface