VASCULAR RESPONSES AND WOUND REPAIR IN MICE EXPOSED TO MODERATE AND SEVERE HYPOXIA

RESEARCH REPORT

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FOREWORD

The present investigation was supported by Public Health Service Grant No. GM-05112 from the National Institute of General Medical Sciences, National Institutes of Health, Bethesda, Maryland. The work was also aided in part by NASA Grant NGR 39-009-015(2).

This report was prepared by Diana R. Godish and was submitted to the Graduate School in partial fulfillment of the requirements for the degree of Doctor of Philosophy at The Pennsylvania State University. The research was conducted by Mrs. Godish as a subproject of a research project entitled "Biophysical and Endocrine Changes in Acclimated Rats" with Dr. Adam Anthony, Professor of Zoology, acting as the project director and Dr. G. K. Strother, Associate Professor of Biophysics as coinvestigator.

The senior author wishes to express her sincere appreciation to Dr. Adam Anthony for guidance and encouragement throughout the research work. She is also grateful to Mr. John Kmetz and Mrs. Athleen Stere for their helpful assistance and to Mr. Edward Prokop who designed and constructed the electrical lesioning apparatus. Appreciation is further expressed to the National Institutes of Health for Graduate Fellowship support in 1965-1966 and 1966-1967.

The experiments reported herein were conducted according to the principles outlined in the "Guide for Laboratory Animal Facilities and Care" and adopted by the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

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INTRODUCTION

It is well established that exposure of an animal to hypoxia, such as would be encountered under reduced barometric pressure, is associated with a complex of physiological responses which aid in survival and eventual adaptation to a rarefied atmosphere. Some of the most prominent alterations are those of the cardiovascular system which are associated with a restoration of oxygen supply to body tissues. These include an increase in blood volume (Anthony and Kreider, 1961), hematocrit (Johnson and Feigen, 1962), hemoglobin concentration (Hurtado, Merino and Delgado, 1945), and bone marrow volume (Hunt and Schraer, 1965) as well as a proliferation of blood vessels throughout the body (Kreider, 1960; Valdivia, 1956).

Although systemic vessel proliferation is known during the first week of hypoxia-exposure (Kreider, 1960), no information is available on the precise pattern of vascular responses which occur in peripheral or internal body regions. Also, little is known about possible relationships between hypoxia-induced angiogenesis and the wound repair potential of various body regions.

The aim of this study, therefore, is to investigate (1) the peripheral and internal vascular response to moderate (380 mm Hg) and severe (320 mm Hg) hypoxia and (2) the relationship of the vascular response to the healing of peripheral and internal wounds.

GENERAL CONSIDERATIONS

A. Review of the Literature

1. Cardiovascular responses to hypoxia

Although there is a wealth of literature dealing with cardiovascular responses associated with hypoxia, very little information is available on hypoxia-induced angiogenesis. Nevertheless, a review of the overall cardiovascular adaptations which occur in response to oxygen deprivation provides insight into some of the complex interactions which enable animals to adjust to oxygen lack. The significance of any changes in blood vessels, and the effect of such changes on wound healing, most ultimately be interpreted in light of the total systemic response.

The net result of successful adaptive cardiovascular alterations is a restoration of oxygen supply to body tissues. Green (1965) has suggested that since the amount of oxygen available in each unit of blood is reduced in hypoxia, this restoration can be only achieved by an increase in the quantity of blood flowing through tissues per unit time. Three processes contribute to any increase: (1) a greater cardiac output; (2) an increase in total circulating red cell volume; and (3) an altered distribution of available blood during acute hypoxia, so that vital tissues are supplied at the expense of less vital regions during this critical period. The contribution of each mechanism depends upon both severity and duration of hypoxia.

Numerous reports of increased cardiac output shortly after the onset of hypoxia are found in the literature (Asmussen and Chiodi, 1947; Asmussen

and Consolazio, 1941; Baugh, Cornett and Hatcher, 1959; Daly and Scott, 1964; Eckenhoff et al., 1947; Fishman et al., 1952; Gorlin and Lewis, 1954; Grollman, 1930; Nahas et al., 1954b; Rahn and Otis, 1947). In contrast, Motley et al. (1947) reported a decrease in cardiac output within a few minutes after administering a mixture of 10 percent oxygen in nitrogen. This difference may be the result of (1) varying experimental degrees of hypoxia and/or (2) the length of time elapsed between hypoxia onset and initial measurements. Fishman (1961) has suggested that inspired mixtures of less than 11-12 percent oxygen are apt to be poorly tolerated, with some variability found among species. When more than 12 percent oxygen is present, he reported a consistent increase in cardiac output of 10-20 percent. Nahas, Visscher and Haddy (1954) have questioned the use of Fick' s method of cardiac output determination when used during the first 5-10 minutes of hypoxia. It has since been shown that determinations made using Stewart-Hamilton' s dye-dilution method agree with results obtained by the Fick method only after an animal has been exposed to hypoxia for a minimum of 20-30 minutes (Fishman, 1961).

The first readily-detectable circulatory response to hypoxia is an increase in pulse rate (Asmussen and Chiodi, 1947; Glick, Plauth and Braunwald, 1964; Grollman, 1930; Nahas et al., 1954b). Since stroke volume is unaltered at this time (Korner, 1959; Nahas et al., 1954b; Wiggers, 1941), the reported augmented cardiac output must be due to an increased heart rate. Within a few hours, pulse rate returns to normal (Rahn and Otis, 1947), stroke volume increases (Korner, 1959) and cardiac output thus remains elevated (Rahn and Otis, 1947). As more permanent adaptive alterations develop, cardiac output gradually returns to normal levels (Asmussen and Consolazio, 1941; Rotta et al., 1956).

Several mechanisms have been proposed to account for the early rise in cardiac output. Decreased peripheral resistance produced by arteriolar dilation, coupled with increased venous return secondary to systemic veno-constriction, has been frequently postulated (Baugh et al., 1959; Harrison et al., 1927; Kahler et al., 1962; Nahas, Josse and Muchow, 1954). Adrenal gland involvement in these vascular changes is still disputed (Baugh et al., 1959; Kahler et al., 1962). A direct stimulatory effect of low oxygen tensions on heart muscle as proposed by Sands and DeGraff (1925), is no longer considered a likely mechanism.

An increase in the hemoglobin concentration of blood results from all of the various mechanisms which allow blood to transport an increased oxygen supply. Initial hemoconcentration in response to hypoxia-exposure has been reported to be due to a reduction in plasma volume (Asmussen and Consolazio, 1941; Brown, Hopper and Wennesland, 1957; Feigen and Johnson, 1964; Fryers, 1952; Lawrence, 1955; Pugh, 1964; Reissman, 1951; Reynafarje, 1957; Van Liere and Stickney, 1963) rather than to cell mobilization. Brown et al., (1957) speculated that this plasma loss is due to increased venule pressure associated with acute hypoxia. This, in turn, by influencing capillary pressure, would cause movement of blood fluid from vascular to extravascular spaces. An alternate or supplementary mechanism for plasma loss has been presented by Badger and Pace (1962). They feel that increased pulmonary venous pressure activates left atrial stretch receptors, with a resultant decrease in ADH secretion.

Direct results of reduced plasma volume during acute hypoxia are: (1) decreased total blood volume (Asmussen and Consolazio, 1941; Feigen

and Johnson, 1964; Reynafarje, 1957) and (2) hemoconcentration (Lawrence, 1955; Reissman, 1951).

By the end of the first week of hypoxia-exposure, blood volume returns to normal (Reissman, 1951); thereafter it gradually increases until a new "steady state" level is reached (Fryers, 1952; Lawrence, 1955; Reissman, 1951; Reynafarje, 1957; Rotta et al., 1956). The rise in blood volume is due to partial recovery of plasma volume over an extended period of time (Pugh, 1964; Reynafarje, 1957) and to an increase in red blood cell volume shortly after the onset of hypoxia (Asmussen and Consolazio, 1941; Chiodi, 1949; Fryers, 1952; Johnson and Feigen, 1962; Lawrence, 1955; Pugh, 1964; Reynafarje, 1957; Rotta et al., 1956). A rise in hematocrit from a normal of near 40 percent to highs of 70-80 percent has been reported to be associated with the increase in red cell volume (Chiodi, 1949; Clark and Otis, 1952; Fryers, 1952; Johnson and Feigen, 1962). The importance of this lies in the 50 percent increase in oxygen-carrying capacity of blood associated with an hematocrit of 85 percent (Clark and Otis, 1952). A major increase in hemoglobin concentration also occurs (Asmussen and Consolazio, 1941; Chiodi, 1949; Feigen and Johnson, 1964; Fryers, 1952; Hall and Barker, 1954; Johnson and Feigen, 1962; Reissman, 1951; Reynafarje, 1957; Rotta et al., 1956) and has been reported to be as great as 30 percent (Pugh, 1964).

Stimulation of erythropoiesis is thought to be due to a circulating humoral factor, erythropoietin, which many workers feel is produced by the kidney (Lucarelli et al., 1963; Naets, 1963). Blood plasma of animals exposed to hypoxic conditions has been found to stimulate production of

both hemoglobin and red blood cells when injected into normal controls (Gordon et al., 1959). Erythropoietic activity is directly related to hypoxia severity (Gurney, 1964; Stohlman and Brecher, 1957). Plasma from man (Reynafarje et al., 1964), guinea pig (Jepson and Lowenstein, 1964; Stohlman and Brecher, 1959) and rat (Prentice and Mirand, 1961) shows the greatest erythropoietic activity after 24 hours of hypoxia-exposure, with such activity essentially disappearing by 72 hours. Stohlman and Brecher (1959) have reported that in cases of chronic hypoxia, the level of circulating erythropoietin is dependent upon severity of hypoxia and state of bone marrow function. Since erythropoietin levels decrease only slowly when marrow hypoplasia is coupled with hypoxia (Stohlman, 1959; Stohlman and Brecher, 1959), it has been suggested that erythropoietin may be removed from circulation by bone marrow. Since hematocrit and total blood volumes were not reported by these workers, it is impossible to determine whether the primary cause of prolonged high erythropoietin levels was the failure of marrow to remove erythropoietin or the continued hypoxic condition of the blood due to little or no increase in red blood cells or hemoglobin concentration. Since erythrocyte production is a function of marrow (Ham and Leeson, 1961) it is possible that erythropoietin has some direct effect on this tissue. Recent work using cultures of rat (Krantz et al., 1963) and human (Krantz, 1965; Necheles, Sheehan and Meyer, 1965) bone marrow cells has shown that erythropoietin provokes a prolonged increase in the rate of heme synthesis in vitro. Necheles et al. (1965) have also found a marked stimulation of globin synthesis in vitro as measured by glycine-C^{14} incorporation.

Although initial hemoconcentration and increased cardiac output serve to partially restore and maintain adequate oxygen supplies to body tissues, they are not sufficient in themselves to fully support the requirements of metabolically active and oxygen-dependent organs such as brain and heart. Both organs are completely dependent upon their immediately-available oxygen supply, and their continued functioning requires large quantities of oxygen. Evidence has accumulated to show preferential shunting of available blood during the acute stages of hypoxia. Several workers have found that severe hypoxia results in increased cerebral blood flow (Courtice, 1941; Wiggers, 1941). Kety and Schmidt (1948) reported that exposure of normal young men to a 10 percent oxygen in nitrogen mixture results in an almost immediate 35 percent increase in blood flow to the brain. Opitz and Schneider (1950) postulated that the degree of cerebral vasodilatation may be best equated to changes in venous pO_2 below a certain critical level.

Coronary vasodilation with a resultant increase in coronary blood flow has also been repeatedly found with reduced oxygen intake (Eckenhoff et al., 1947; Feinberg, Gerola and Katz, 1958; Haeckel and Clowes, 1956; Katz, 1958). It has been proposed that coronary vasodilatation is due primarily to the influence of some local metabolite; this is supported by the finding that flow regulation in hypoxia is not appreciably different from flow regulation under normal oxygen conditions (Haeckel and Clowes, 1956; Feinberg et al., 1958).

That blood is preferentially shunted from peripheral regions to more vital organs is further substantiated by the work of Schneider and Truesdell

(1924) and Abramson, Landt and Benjamin (1943) who reported that during acute hypoxia the hand shows vasoconstriction as well as reduced blood flow volume. Similar results have been shown using skin of anaesthetized and unanaesthetized dogs (Rein, Loose and Otto, 1941). In addition, blood flow is reportedly decreased in dog muscle under 5-6 percent inspired oxygen (Schroeder, Schoop and Stein, 1954). Studies on circulation through visceral regions have been inconclusive. Observations on isolated intestinal loops in hypoxic animals (1 percent oxygen in nitrogen) have revealed a definite vasoconstriction (Bernthal and Schwind, 1945). On the other hand, if 7-10 percent oxygen were employed, net blood flow through perfused intestine was normal (Bean and Sidky, 1957). Unfortunately, no estimates of total splanchnic flow during prolonged hypoxia are available (Korner, 1959). Being a vital functional body region, it would be expected that circulation be augmented during hypoxia.

Acclimation to hypoxia demands that all body tissues receive quantities of oxygen adequate for the sustenance of life and for additional growth. Vascular hyperplasia is a major factor in accomplishing ultimate acclimation, since the presence of additional capillaries in tissue serves to cut down on diffusion distances between blood vessel and cell (Korner, 1959; Van Liere and Stickney, 1963). Such an increase in the number of blood vessels per unit volume of tissue in response to prolonged hypoxia has been reported in skeletal muscle (Valdivia, Ottensmeyer and Davis, 1962), myocardium (Hurtado, 1960), eyes (Opitz, 1951), brain (Pawel, Clark and Chinn, 1954) and internal systemic organs (Anthony and Kreider, 1961).

The adequacy of cardiovascular adjustments to hypoxia described above is best illustrated by reports of several independent investigators

that the rate of oxygen consumption remains unchanged when the partial pressure of oxygen is reduced within tolerable limits (Clark and Otis, 1952; Houston and Riley, 1947). This implies that the amount of oxygen available to tissues following acclimation is the same as was available prior to hypoxia, and therefore indicates that adaptive alterations in the cardiovascular system have been successful in maintaining internal homeostasis.

2. Wound healing in hypoxic animals

Normal healing processes are apparently intimately dependent upon blood supply. Vascular proliferation in the wound area is known to be one of the sequential steps in the repair of any body injury (Perez-Tamayo, 1961).

The literature provides little information on relationships which may exist between hypoxia-exposure and the healing process. Weihe (1964) studied the effect of acute hypoxia on the healing rate of dorsolumbar skin wounds in rats and found that it was decreased. Differences due to species and strain were reported. His findings are similar to those of other workers (Utinka, 1958). It must be pointed out, however, that no work has been carried out using internal wounds or wounds induced following acclimation.

Since the vascular responses of such regions as skin and visceral tissues may differ during acute hypoxia (as discussed above), it is of considerable interest to investigate the healing potential of these respective regions during both acute and chronic hypoxia.

B. Restatement of the Aims

The nature of the vascular response of peripheral and internal body

regions of the mouse to moderate and severe hypoxia, and its relationship to wound healing, was examined in this study. Specifically, the aims were as follows:

1. To investigate the nature of peripheral vascular changes which occur during acute and prolonged hypoxia-exposure and to determine how these effect the healing of peripheral body wounds. This involved:
   (a) quantitative and microscopic analysis of the external ear vasculature as a function of exposure time,
   (b) study of repair of induced ear wounds (ca 2 mm^2 ear puncture) and
   (c) comparison of the effects of severe (320 mm Hg) and moderate (380 mm Hg) hypoxia on both the vascular response and healing of the external ear.
2. To investigate internal vascular changes which occur during hypoxia-exposure in relation to the healing of induced fundic stomach wounds. This involved histological, histochemical and cytological analysis of tissue repair following production of a small (ca 0.7 mm^2) burn in the submucosa by electrocautery.

MATERIALS AND METHODS

A. Experimental Animals and the Decompression Chamber

A total of 234 adult male albino mice (A/HeJ strain, Jackson Laboratory, Bar Harbor, Maine) weighing approximately 23 ± 2 grams served as subjects for this study. Except as later indicated, they were maintained on tap water and Purina Lab Chow ad libitum.

The altitude chamber used has been previously described (Anthony, Ackerman and Strother, 1959). It consisted of an animal exposure compartment and a smaller walk-in air lock compartment which permitted continuous maintenance of animals at the desired simulated altitude during cleaning and feeding operations. The animal chamber had a volume of 286 cubic feet. The turnover rate of air was estimated to be about eight cubic feet per minute at a pressure of one-half atmosphere.

All hypoxic mice used in this study were maintained at a pressure of either 380 ± 10 mm Hg or 320 ± 10 mm Hg. These pressures correspond to simulated high altitudes of approximately 18,000 ± 500 feet and 22,000 ± 500 feet above sea level respectively (Armstrong, 1939). Control mice were kept at ground level in University Park, Pennsylvania (725 mm Hg or 1200 feet).

B. General Experimental Procedure

The study was composed of three major related experiments which investigated the effect of altitude hypoxia on:

1. the peripheral vascular response and healing of external ear wounds,
2. the internal vascular response and healing of fundic stomach wounds, and
3. hematocrit, organ weights and body weight.

The number of mice used in each study and the various experimental conditions employed are summarized in Table 1.

Techniques followed in each experiment as well as the type of data obtained are discussed below.

1. Peripheral vascular response and healing of external ear wounds in hypoxic mice

One-hundred-thirty-five mice were separated into seven experimental groups. Twenty-five mice were exposed to a reduced pressure of 370 ± 10 mm Hg for 4 to 12 days in an exploratory study to determine the exposure time necessary to elicit grossly apparent alterations in the ear vasculature. The ear was chosen for study because of the relative ease of detailed observation. Initial observations were made through a window in the decompression chamber using a magnifying lamp (1.25x); subsequent examination of the animals after their return to ambient conditions revealed that any hypoxia-induced changes in ear vasculature were stable for a short time. Such stability increased with increasing length of hypoxia exposure. It was therefore felt that detailed examination of vascular changes could validly be conducted under ambient conditions if such examination were completed within 3 to 4 hours after removal of the animal from an hypoxic condition. Autopsies were performed at 4-day intervals to check on the vascular state of internal organs.

TABLE 1. EXPERIMENTAL DESIGN

Group	Number of mice	Experimental treatment
I. Peripheral vascular response and healing of external ear wounds in male A/HeJ mice (total N = 135). *		
1 Exploratory	25	370 mm Hg for 0 to 12 days
2 Control	23	725 mm Hg; fed ad libitum
3 Control	5	725 mm Hg; limited food intake on days 0 to 13
4 Severe hypoxia	20	Wounded day 0 of exposure to 320 mm Hg
5 Moderate hypoxia	29	Wounded day 0 of exposure to 380 mm Hg
6 Severe hypoxia	13	Acclimated to 320 mm Hg for 19 days prior to wounding and maintained under hypoxia
7 Moderate hypoxia	20	Acclimated to 380 mm Hg for 19 days prior to wounding and maintained under hypoxia
II. Internal vascular changes and healing of fundic stomach wounds in male A/HeJ mice (total N = 71). **		
1 Exploratory	27	725 mm Hg at all times
2 Control	3	Unwounded; maintained at 725 mm Hg
3 Control	7	725 mm Hg; limited food intake on days 0 to 14
4 Control	9	725 mm Hg; fed ad libitum
5 Severe hypoxia	10	Wounded day 0 of exposure to 320 mm Hg
6 Severe hypoxia	6	Unwounded; exposed to 320 mm Hg for 6, 12 or 19 days
7 Moderate hypoxia	9	Wounded day 0 of exposure to 380 mm Hg

*Observations of the vasculature were made on days 0, 12, 19, 26, 33 and 40 of hypoxia exposure. Ear punctures were allowed to heal 21 days prior to termination of an experiment.

**In all except groups 2 and 6, animals received two fundic lesions. Mice were sacrificed 6, 12 or 19 days after wounding.

TABLE 1. EXPERIMENTAL DESIGN (Continued)

Group	Number of mice	Experimental treatment
III. Hematocrit, organ weight and body weight changes in male A/HeJ mice (total N = 94). ***		
A. Hematocrit studies (total n = 60).		
1 Control	24	725 mm Hg at all times
2 Severe hypoxia	18	320 mm Hg for 40 days
3 Moderate hypoxia	18	380 mm Hg for 40 days
B. Organ weight studies (total n = 66).		
1 Control	21	725 mm Hg at all times
2 Severe hypoxia	15	320 mm Hg for 21 days
3 Severe hypoxia	6	320 mm Hg for 40 days
4 Moderate hypoxia	12	380 mm Hg for 21 days
5 Moderate hypoxia	12	380 mm Hg for 40 days
C. Body weight studies (total n = 70).		
1 Control	28	725 mm Hg at all times
2 Severe hypoxia	24	320 mm Hg for 40 days
3 Moderate hypoxia	18	380 mm Hg for 40 days

***Hematocrit and body weight measurements were made on days 0, 12, 19, 26, 33 and 40 of hypoxia exposure. The weights of thymus, adrenals, spleen, heart, testes and seminal vesicles were determined on days 21 and 40; in the case of 21-day exposed animals (groups B-2 and 4) initial and terminal body weights were also recorded.

The six remaining groups of mice were treated as indicated in Table 1. Animals wounded under acute hypoxia were wounded on day 0 of exposure and maintained at reduced pressure for 21 days; those wounded under chronic hypoxia were wounded on day 19 of exposure and kept at reduced pressure for 21 additional days. Control mice remained at ambient barometric pressure at all times. Since mice undergo a voluntary reduction in food intake on exposure to hypoxia (Isenberg, 1966), five control mice were maintained on a reduced food intake throughout the first 13 days after wounding to determine the effect of limited dietary intake on the healing process.[1]

The wound in all cases was a hole (1.88 mm^2 area) punched in the right pinna (i.e. external ear) of each mouse with a small paper punch. The hole was produced in the central region of the pinna; care was taken to minimize the number of blood vessels damaged, and no major vessels were ever broken.

Two different parameters were studied during this phase of the experiment. First, counts were made of the number of venous ramifications down to the terminal venules in both intact and punctured ears of eight mice from each of groups 2 and 6 and three mice from group 7. This was done on anaesthetized animals (pentobarbital, 0.06 mg/gm) using a Bausch and Lomb stereozoom microscope (15x) on days 0, 12, 19, 26, 33 and 40 of the experiment to establish the effects of hypoxia on the

[1]Dietary regimen of ambient pressure controls maintained on limited food intake is as follows with first number indicating day of the experiment and that in parenthesis the average amount of Purina lab chow eaten in grams/mouse: 0 (3.1); 1 (2.0); 2 (1.6); 3 (2.3); 4 (2.1); 5 (1.7); 6 (2.0); 7 (1.7); 8 (2.9); 9 (2.6); 10 (2.4); 11 (2.6); 12 (2.5); 13 (2.7); 14 (2.9).

extent and rate of peripheral angiogenesis. Counts were based on detailed drawings of the ear vasculature made at these times. The ear being drawn was held gently between two microscope slides separated by a shorter standard cover slip; this arrangement permitted a flat view of the entire ear while at the same time allowing maintenance of normal circulation.

The second measurement compared peripheral healing rates in mice acutely exposed to moderate (380 mm Hg) or severe (320 mm Hg) hypoxia. Comparison was also made of healing in moderately and severely hypoxic mice acclimated 19 days prior to wounding. Twenty-one days after wound induction, measurements of the wound were made under a 15x microscope using a ruler divided into 0. 5 mm units. The extent of healing was expressed as percentage of ear puncture filled with granulation tissue. The aims of this latter phase were to establish: (1) whether healing was retarded during the acute stage of hypoxia, (2) if adaptive changes in vasculature entailed a restoration of healing potential and (3) whether the degree of hypoxia severity affected either (1) or (2) above.

Five mice were employed for a check of the peripheral arterial response to hypoxia. Three animals were exposed to 380 mm Hg pressure for 40 days (group 7) while two mice served as controls (group 2). They were handled in the same way as groups 2 through 7 above, but arterial ramifications to the level of small arteries were studied. This was done to determine the extent of parallelism between hypoxia-induced changes of the peripheral venous and arterial vasculatures.

Supplemental data were obtained from microscopic analysis of 10 percent formalin-fixed ear sections processed using the paraffin method,

sectioned at 6 μ and stained with Lillie' s Allochrome (Gurr, 1960).

2. Internal vascular response and healing of fundic stomach wounds in hypoxic mice

Seventy-one mice were separated into exploratory and experimental groups (Table 1).

Twenty-seven animals, maintained at ambient conditions, were employed to determine: (1) the most satisfactory organ and region thereof to be injured, (2) the best method of inducing injury and (3) specific techniques to be used therein. These studies resulted in adoption of the procedure which was employed in all subsequent experiments.

Wounds were lesions produced by a pair of machine grade mild steel electrodes partially embedded in a leucite plastic holder with their tips approximately 0.3 mm apart. A DC amperage of 0.8-2.0 mamps was maintained with a Hewlett Packard DC power supply (Model 721 A) and measured by a DC vacuum tube voltmeter (Hewlett Packard, Model 412 A). Current was applied to the serosa of the fundic stomach for five seconds; this was done on the ventro-lateral aspect of the greater curvature. Damage was limited to the serosa, muscularis externa and submucosa. Each mouse was anesthetized with Nembutol (pentobarbital, 0.06 mg/gm) and a ventral incision approximately 1.5 cm long was made. The fundus was exposed and two adjacent lesions were induced. Care was taken to avoid direct injury to any major blood vessels. Four to five sutures (size 40 cotton thread) were made to close the incision. All wounded animals received their wounds just prior to the onset of any experimental conditions.

Forty-four mice were separated into six groups for the major phase

of this experiment (Table 1). Twenty-five animals were exposed to either moderate (380 mm Hg) or severe (320 mm Hg) acute hypoxia. Six of the severely hypoxic mice received no wounds. Ambient pressure controls were subdivided into three groups: controls fed ad libitum, controls on limited food and water intake during the first 14 days of the experiment, and unwounded controls. The food-limited controls were employed to maintain in hypoxic and control mice an equivalent state of: (1) nutritional intake and (2) wound stress due to fundic stretching (see footnote, page 15). All unwounded animals were used to provide information on normal fundic tissue under ambient and reduced pressure conditions.

At the termination of a given exposure period (i.e. 6, 12 or 19 days), animals were sacrificed by ether anesthesia. The entire area of tissue surrounding the two wounds was excised and fixed in 10 percent phosphate-buffered formalin for subsequent histological examination. Following routine paraffin embedding, tissues were cut serially at 8 μ and alternate slides were stained by either toluidine blue 0 (Gurr, 1960) or the Picro-Gomori method (Humason, 1962).

The wound area was studied histologically using the following criteria as indices of the degree or extent of inflammation and healing: (1) gross appearance of the healing wound, i.e. extent of tissue organization and extent of mesothelial regrowth, etc., (2) differential cell counts in (a) submucosa adjacent to the wound area and (b) the wound itself (cells classed as (i) fibroblasts and fibrocytes; (ii) macrophages, i.e. neutrophils, lymphocytes, phagocytes, etc.; or (iii) mast cells), and (3) degree of metachromatic staining with toluidine blue 0. Vascular changes of the fundic

region in all groups were observed using tissues stained by the Picro-Gomori method.

3. Hematocrit, organ weight and body weight changes

Corollary information on selected aspects of the physiological response to altitude hypoxia was also obtained. Animals used were primarily those involved in the study of peripheral vascular changes and healing. The number of animals in each group may be found in Table 1.

Blood samples were taken from the postorbital sinus of the eye (Pansky et al., 1961) using 32 x 0.8 mm heparinized capillary tubes. The micro-hematocrit tubes were flame-closed at one end, centrifuged in an International micro-capillary centrifuge, model MB, and then read on a Critocap micro-hematocrit tube reader.

Body weights were recorded each time an animal was handled, i.e. days 0, 12, 19, 26, 33 and 40 of hypoxia exposure. Animals were weighed on a standard beam balance (Central Scientific Co., Chicago). At the termination of an experiment (day 21 or 40) animals were sacrificed by ether anesthesia. Fresh weight of the thymus, spleen, heart, adrenal, testes and seminal vesicles was determined on a Roller Smith torsion balance and recorded. These organs were fixed in a 10 percent phosphate-buffered formalin solution and saved for possible histological studies.

RESULTS

The major findings are summarized in three subsections: A. peripheral vascular response and healing of ear wounds in hypoxic mice, B. internal vascular response and healing of fundic wounds in hypoxic mice and C. hematocrit, organ weight and body weight changes during hypoxia exposure.

A. Peripheral Vascular Response and Healing of Ear Wounds in Hypoxic Mice

1. Gross observations

During the first few days of hypoxia-exposure mice remained relatively inactive and exhibited no detectable alterations in ear vasculature. By day 4 a moderate hyperemia was evidenced in the ears of all hypoxic mice. This became progressively more pronounced after 8 and 12 days. Closer examination of ears revealed that hyperemia was due primarily to dilatation of major veins rather than to newly formed vessels, thus indicating a reduced venous return from peripheral integument. Hyperemia was more evident in severely hypoxic mice than in those exposed to moderate hypoxia. It was further observed that the greatest increase in ear vascularity occurred during the third and fourth weeks of exposure. The extent of hyperplasia was essentially parallel in both arterial and venous systems. In addition to noting the occurrence of venous dilatation early in exposure, it was also found that ears of hypoxic mice often exhibited varying degrees of epithelial desquamation which appeared as discrete patches of cornification in two-week exposed mice. No difference was ever found between the vascular response of left and right ears.

Thus, gross observation revealed that hypoxia stimulated arterial and venous development to about the same extent in the external ear. In addition, angiogenesis was not markedly evidenced until the third week of exposure.

2. Quantification of ear vascular changes

Since there was an equivalent development of both arterial and venous vasculatures in hypoxia, detailed measurements were limited to the venous pattern, with only periodic checks of the arterial response of representative animals. Terminal venules were more readily discernible than arterioles, providing a more reliable index for quantitating overall vascular response.

A composite illustration of progressive changes in the ear venous network of a mouse exposed to moderate hypoxia for 40 days is presented in Figure 1. The total vascular response of the ear is shown in Figure 2. These demonstrate the marked proliferation of both arterial and venous vasculatures during extended hypoxia.

Quantitative data on ear angiogenesis in mice exposed to severe (320 mm Hg) or moderate (380 mm Hg) hypoxia for 0, 12, 19, 26, 33 and 40 days and in unexposed controls are summarized in Figures 3 and 4. The pattern of venous hyperplasia produced by moderate and severe hypoxia was identical (Figure 3). The most marked vascularization occurred between days 12 and 26. Fewer new ear vessels were evidenced under severe, as compared with moderate, hypoxia on day 12 of exposure. Mice exposed to severe hypoxia had consistently fewer new ear vessels throughout the 40-day exposure period than did moderately hypoxic animals. Arterial response to hypoxia paralleled venous response (Figure 4).

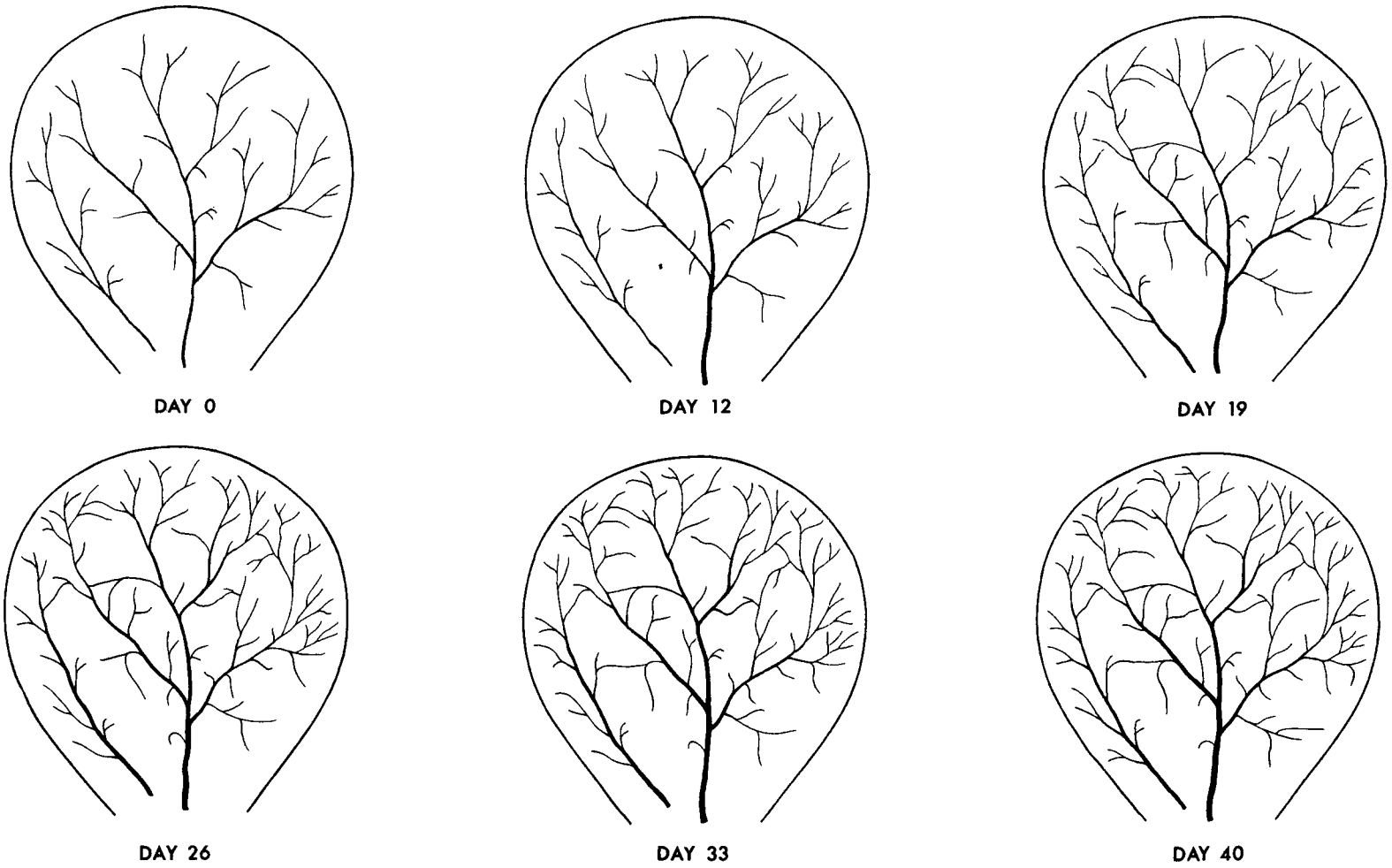

Figure 1. Effect of hypoxia on the venous vasculature of mouse ears.

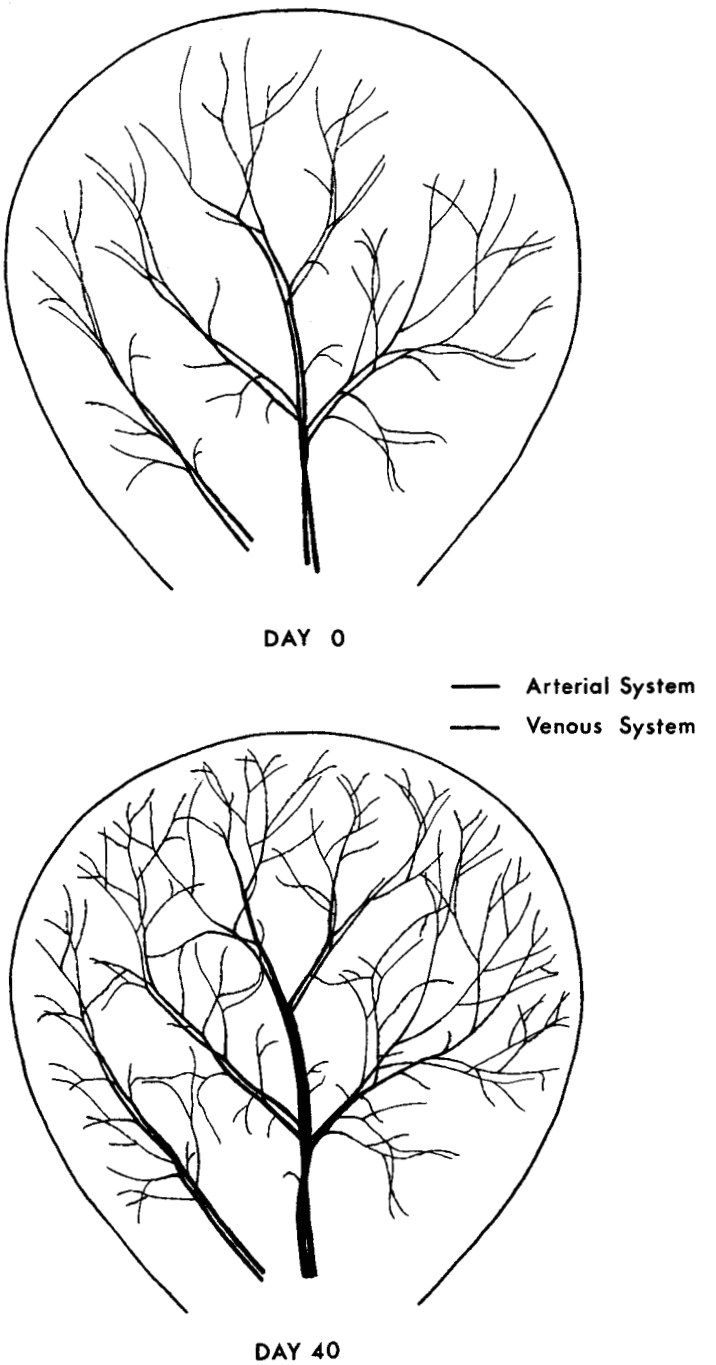

Figure 2. Ear vasculature of mice exposed to prolonged hypoxia (40 days).

Figure 5 summarizes data on the number of ear veins exhibiting increased dilatation. Hypoxic mice had significantly more dilated veins than controls. Dilatation was readily apparent after 12 days of hypoxia and, as in the case of vessel hyperplasia, was most marked between days 12 and 26. This response was similar under either moderate or severe hypoxia although, as previously noted, the degree of dilatation was greater in severely hypoxic animals during the initial two weeks of exposure. Evidence of marked venous dilatation during early hypoxia, when coupled with the previously mentioned absence of new blood vessels, indicated the ear contained a considerable amount of unoxygenated blood. Counts of dilated veins do not reflect the volume of unoxygenated blood since initially only larger veins dilated; then, when the limits of their expansion were reached, smaller veins also expanded.

Microscopic examination of pinnal tissue sections confirmed that vascular hyperplasia was restricted primarily to the network of terminal vessels (arterioles and venules) and capillaries. Arterioles and venules proliferated to about the same extent. That is, for every new vein appearing there was a corresponding artery, although arterioles were not as prominent as venules. From stained sections it was also confirmed that ear veins remained dilated throughout the course of hypoxia-exposure.

In summary, gross and microscopic data demonstrated that two types of vascular changes in ear integument were elicited by hypoxia-exposure: an early venous dilatation indicative of impaired circulation and a subsequent vascular proliferation. The data further indicated that the most marked increase in blood supply to the ear occurred during the third to fourth weeks of exposure as evidenced by a striking vascular hyperplasia. In contrast, during the first two weeks there was only limited angiogenesis.

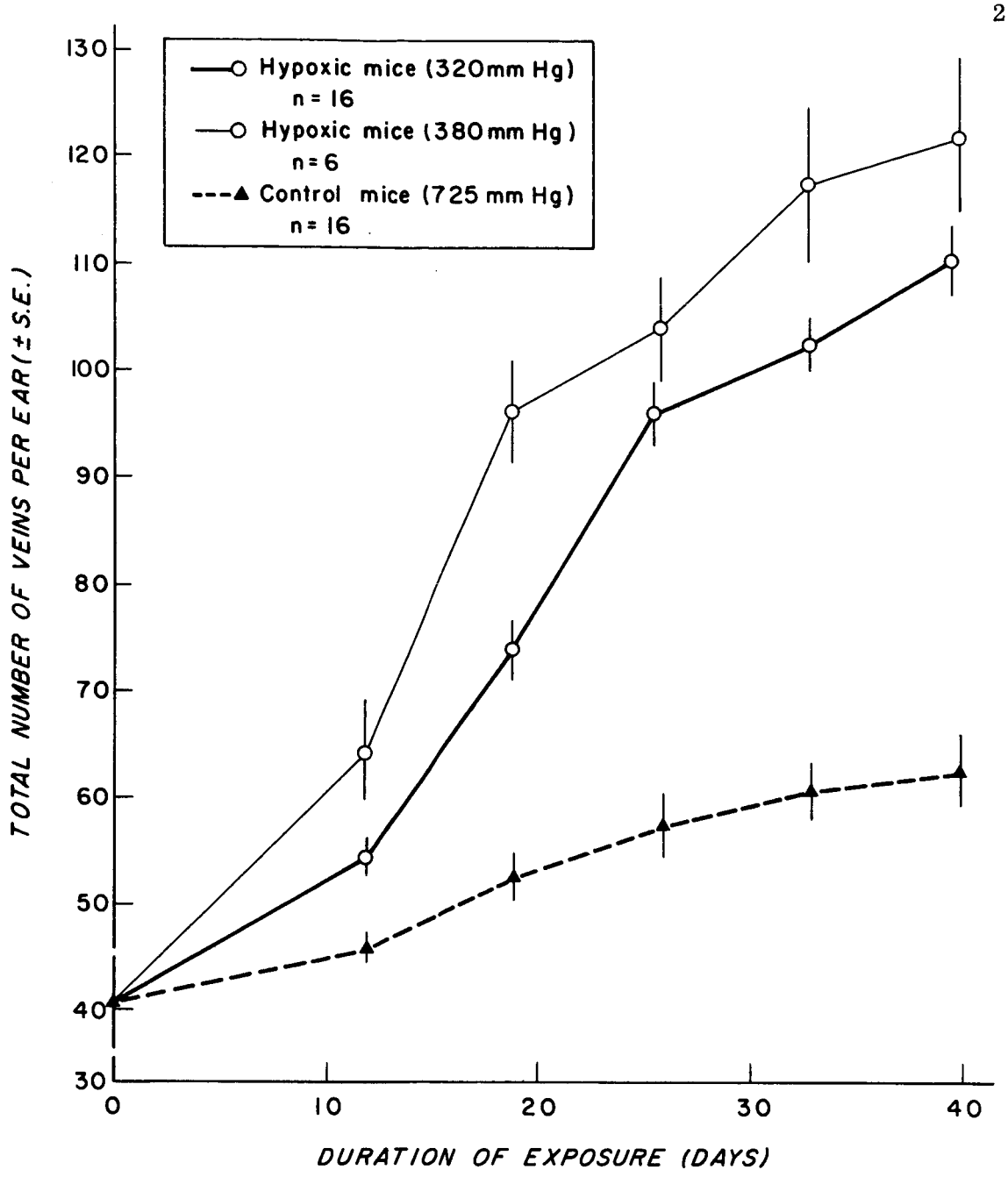

Figure 3. Effect of hypoxia on venous hyperplasia in the mouse ear.

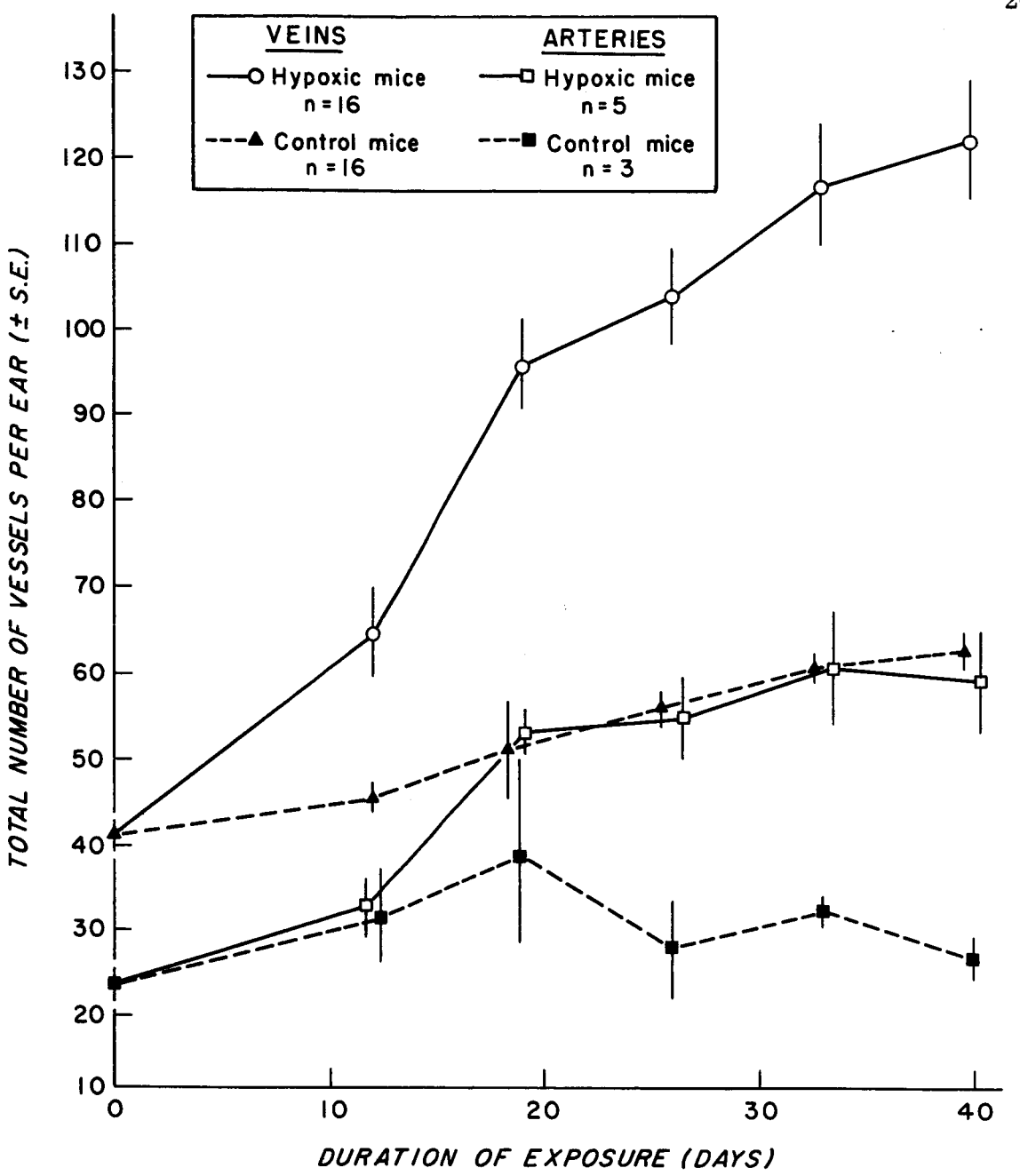

Figure 4. Effect of hypoxia on vascular hyperplasia in the mouse ear (380 mm Hg).

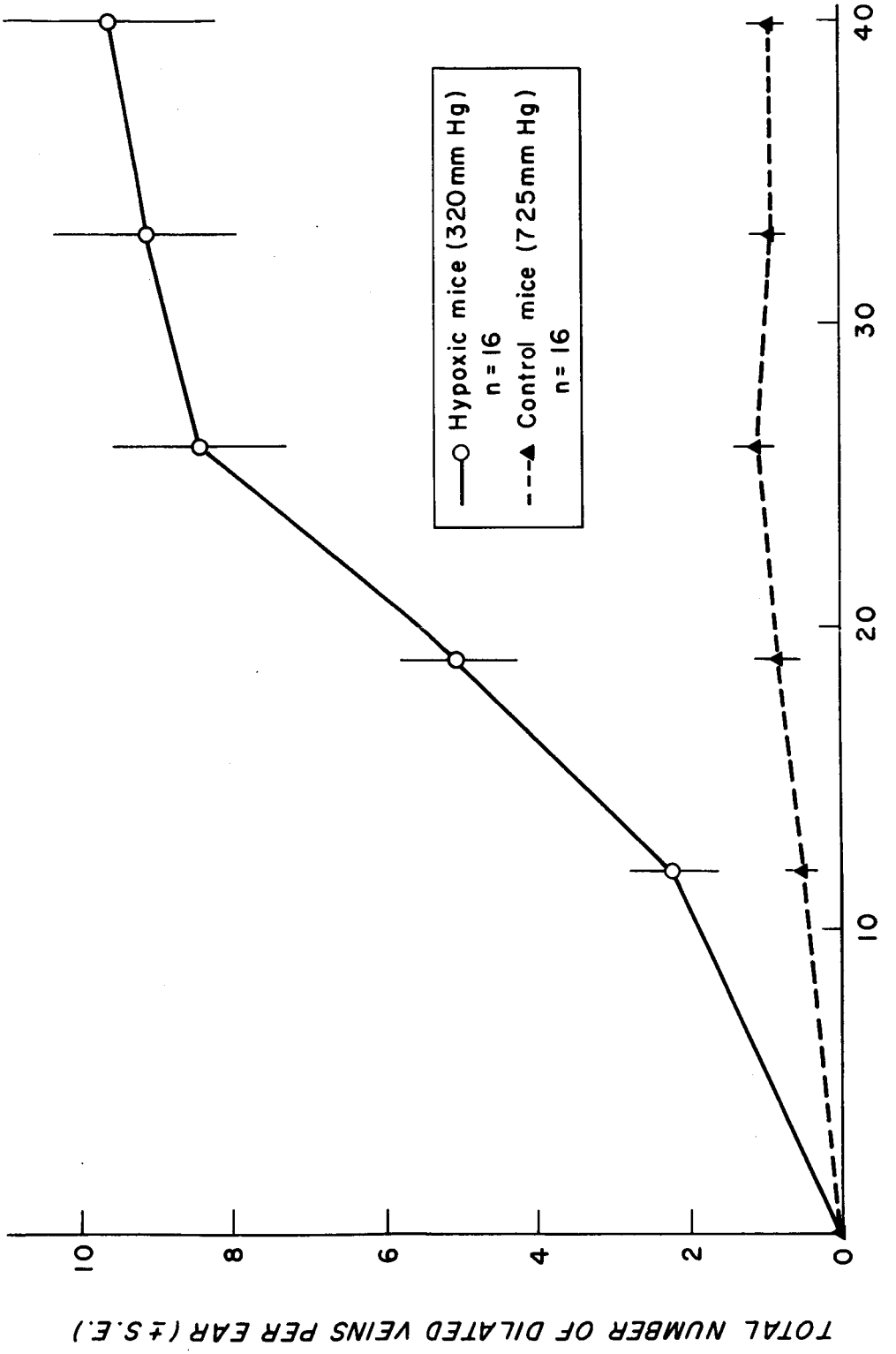

Figure 5. Effect of hypoxia on venous dilatation in the mouse ear.

3. Healing of peripheral body wounds

Data on healing of ear punctures in mice exposed to moderate (380 mm Hg) and severe (320 mm Hg) acute hypoxia or fed a limited dietary intake are summarized in Figure 6. Results on healing in mice with a previous history of hypoxia exposure are summarized in Figure 7. Healing was retarded with severe hypoxia exposure (group A) but not with moderate hypoxia (group B). This was reflected in percentage closure of the wound at the end of a 21-day healing period. Ear punctures in moderately hypoxic and control mice were almost completely healed (93-95 percent) whereas holes in severely hypoxic mice were about seven times larger (only 63 percent closure). Visual inspection of ear punctures at weekly intervals revealed that ears of moderately hypoxic and control mice healed more rapidly than those of severely hypoxic animals throughout the three-week period. Complete closure was first observed on day 21. In all instances, greater venous dilatation was present in the severely hypoxic group than in moderately hypoxic or control groups, confirming earlier results. It was also demonstrated that 13 days of food restriction alone did not affect the healing process (group D).

The effects of prior acclimation on ear wound healing in mice exposed to severe or moderate hypoxia are summarized in Figure 7. Prior acclimation to severe hypoxia restored peripheral wound repair potential but did not enhance peripheral healing in moderately hypoxic animals. No difference in the extent of venous dilatation was detected in the two hypoxic groups; both exhibited more dilatation than controls. As observed in previous studies, the pinnal vascular network was greatly

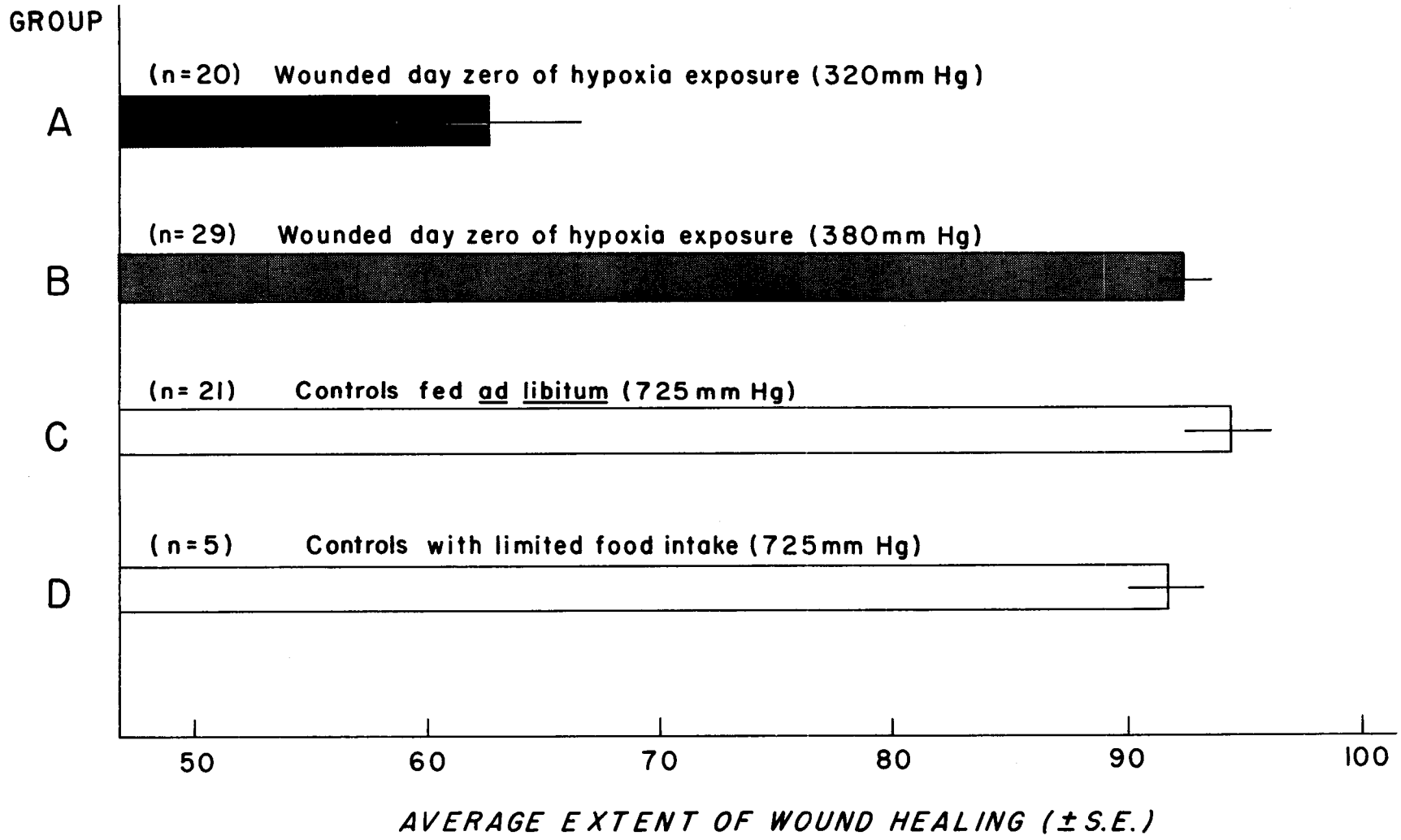

Figure 6. Effect of moderate and severe hypoxia on wound healing in the mouse ear. (Averages expressed as percentage of ear puncture filled with granulation tissue 21 days after wound induction.)

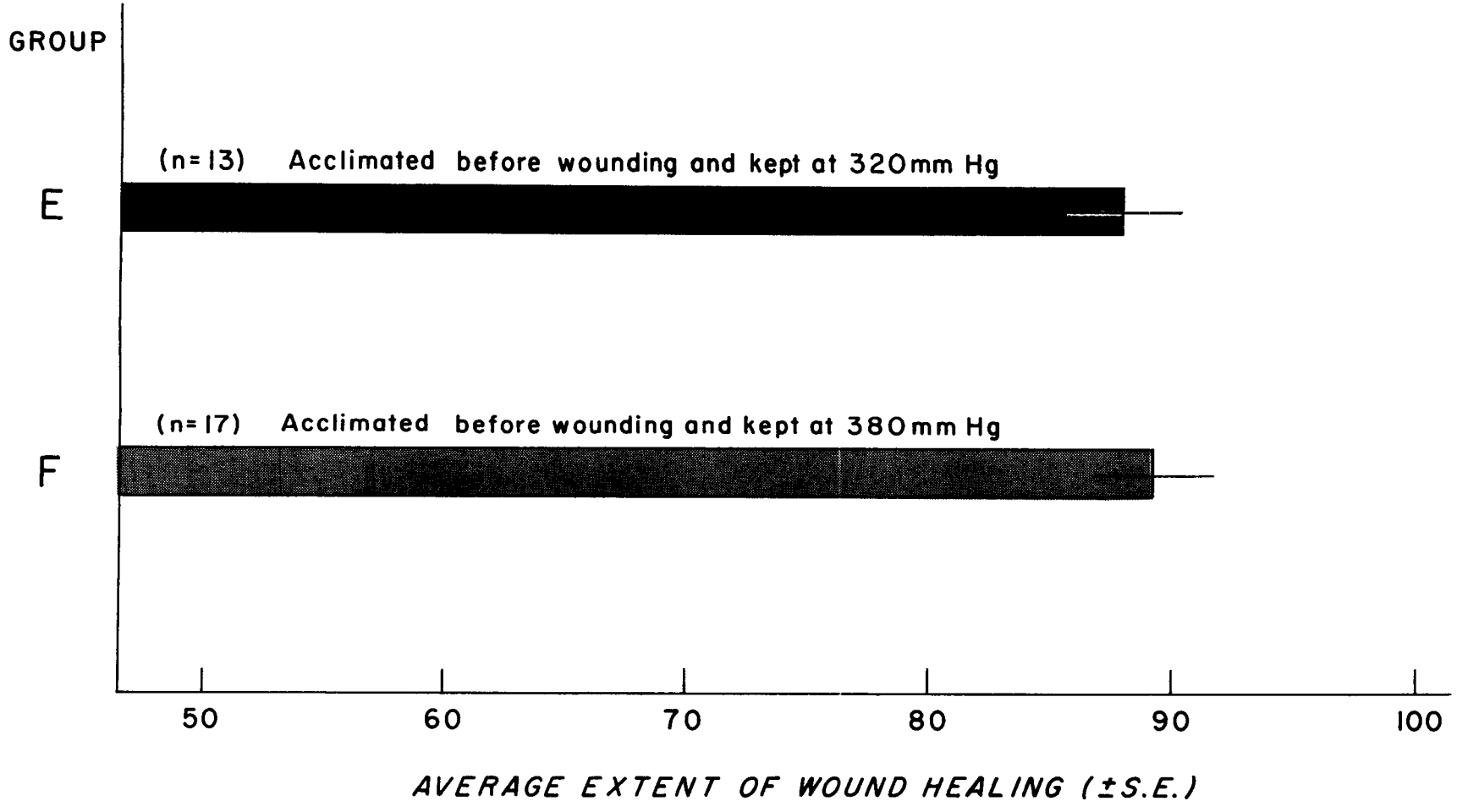

Figure 7. Effect of prior acclimation to hypoxia (19 days) on wound healing in the mouse ear. (Averages expressed as percentage of ear puncture filled with granulation tissue 21 days after wound induction.)

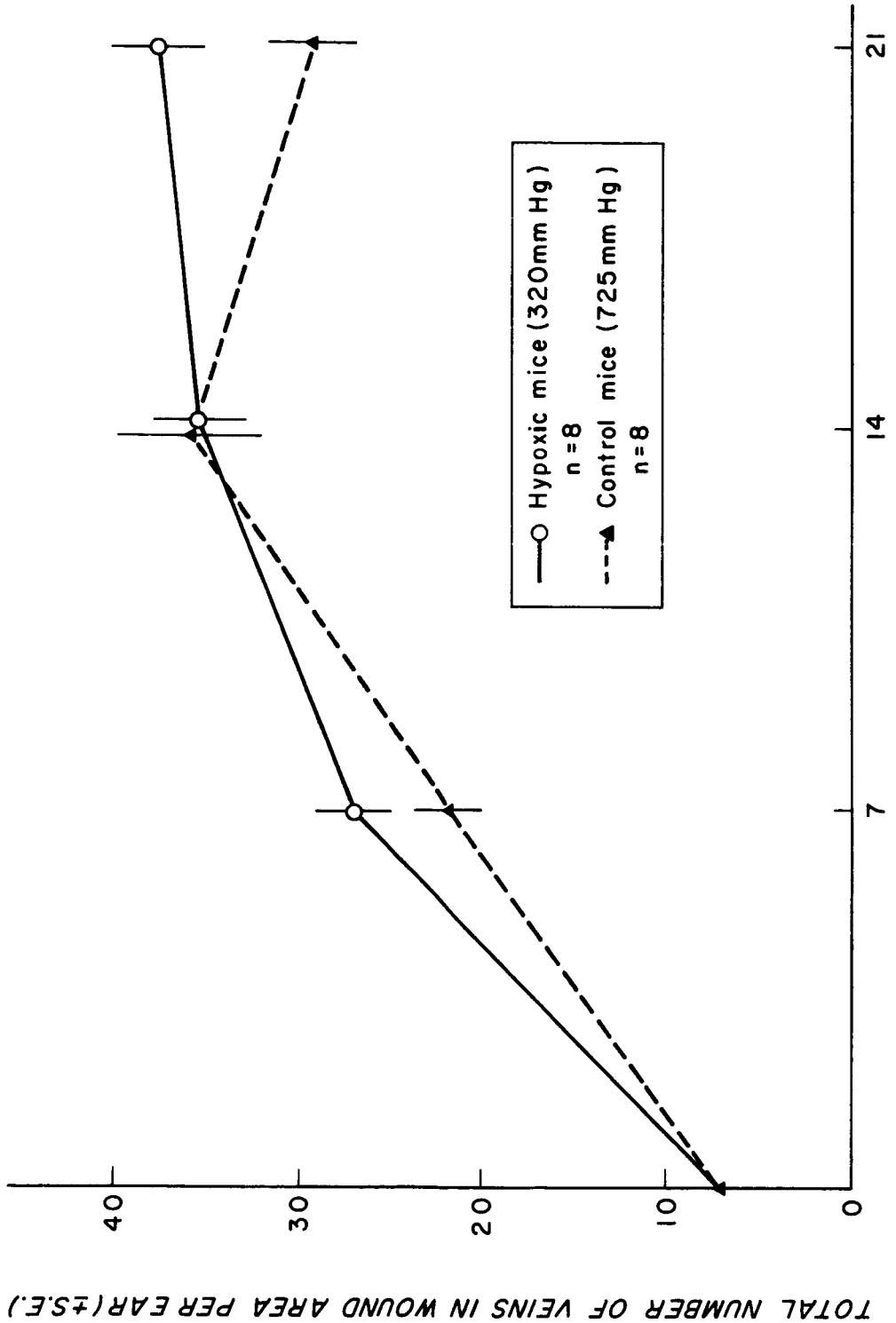

Figure 8. Effect of hypoxia on venous hyperplasia in the wound area (mouse ear). (Hypoxic mice were acclimated for 19 days prior to receiving wound.)

developed in hypoxic mice. In addition, the network immediately surrounding wounds developed to the same extent during the first two weeks post-wounding in both hypoxic and control animals. This is illustrated by Figure 8 which summarizes data on total number of veins per wound area 7, 14 and 21 days after wounding. Reduction in the number of vessels near control wounds on day 21 was attributed to completion of healing in this group.

Briefly, major findings of this phase of work were as follows: (1) healing of peripheral wounds was retarded by severe but not moderate hypoxia, (2) 19 days of acclimation to severe hypoxia resulted in sufficient compensatory vascular adjustments to restore the healing potential to near normal levels and (3) the initiation of healing processes appeared to be dependent upon an hypoxia-induced adaptive increase in the vascular network.

B. Internal Vascular Response and Healing of Fundic Stomach Wounds in Hypoxic Mice

A study was carried out on the internal body vascular response and healing of visceral wounds in acutely hypoxic mice. This was done in an effort to clarify relationships between (1) peripheral vascular response and total body vascular response to hypoxia and (2) vascular response and wound healing.

1. Gross observations

Examination of internal organs 4, 8 and 12 days after the onset of hypoxia-exposure revealed a proliferation of systemic blood vessels by day 4 which became more pronounced with time. Marked venous

dilatation was also present. Although these changes were evident throughout the body cavity they were most pronounced in mesenteries and visceral peritoneum.

Closer inspection revealed several major differences in the vascular appearance of hypoxic mice relative to ambient pressure controls. Hypoxia was associated with the following internal vascular alterations: (1) more extensive branching of blood vessels, (2) dilatation and tortuosity of larger veins and (3) venous congestion and pooling of blood in the visceral region which gave organs a purplish hue. Severance of a large blood vessel in an hypoxic mouse resulted in immediate filling of the abdominal cavity; such massive hemorrhage did not occur in controls.

Microscopic examination of stained fundic stomach sections gave further evidence of early vascular changes. As would be expected with marked tortuosity, more numerous oblique transects of larger vessels were noted. Terminal vessel hyperplasia and venous engorgement were also pronounced.

In summary, both gross and microscopic observation indicated that hypoxia effects a pooling of blood to the viscera within four days of initial exposure. Vascular proliferation was also evident on day 4 and became more pronounced in 8- and 12-day exposed animals. Hyperplasia thus occurred at a much earlier time in internal than in peripheral body regions.

2. Healing of internal body wounds

No differences which could be attributed to hypoxia-exposure were observed in the gross appearance of healing fundic wounds. Histological

features of the wound site in both experimental and control mice are presented in Table 2. There was no indication that hypoxia-exposure or food deprivation markedly affected either the cellular responses associated with healing or the mucopolysaccharide content of ground substance as reflected in metachromatic staining intensity with toluidine blue 0. However, more mast cells occurred in the wound area in starved mice and hypoxic mice which consumed little food.

The response to starvation and hypoxia of submucosal mast cells adjacent the wound area is shown in Figures 9 and 10. Submucosa mast cells were more abundant in hypoxic mice but the number of cells proved to be unaffected by fundic wounding (Figure 9) or hypoxia duration. Mast cell hyperplasia was shown to be due almost entirely to reduced dietary intake associated with the first two weeks of hypoxia exposure.(Figure 10).

In summary, observations made on healing fundic wounds indicated that acute hypoxia had no effect on repair processes in internal body regions. Evidence was obtained from observed similarities in: (1) pattern of fundic healing, (2) presence of blood-borne cells at the wound site (i.e. polymorphonuclear neutrophils, lymphocytes and monocytes) and (3) vascular changes associated with the wound area.

C. Hematocrit, Organ Weight and Body Weight Changes

As a check on certain aspects of the gross physiological state of animals under moderate and severe hypoxia, measurements were made of hematocrit, body weight and weight of selected organs. Findings are summarized below.

TABLE 2. HISTOLOGICAL ANALYSIS OF THE FUNDIC WOUND IN HYPOXIC MICE (320 and 380 mm Hg)[1].

Parameter investigated	Experimental condition	Observations
Vascularity	Controls	Hyperplasia of terminal vessels adjacent to wound by day 6 coupled with some venous dilatation, both increasing with time.
	Hypoxic	Same.
Macrophage to fibroblast ratio (M:F)	Controls (starved)	M:F primarily 1:1 at all times.
	Controls (fed)	Same but with occasional instances of macrophagic infiltration.
	Hypoxic	Same.
Appearance of neo-formed connective tissue	Controls	Orientation of connective tissue elements parallel orientation of normal tissue.
	Hypoxic	Same.
Metachromasia	Controls	Mild metachromasy by day 6, increasing with time.
	Hypoxic	Same.
Appearance of wound surface	Controls	Normal and flat to quite convoluted.
	Hypoxic (320 mm Hg)	Same.
	Hypoxic (380 mm Hg)	Mildly to very convoluted with few normal.

TABLE 2. HISTOLOGICAL ANALYSIS OF THE FUNDIC WOUND IN HYPOXIC MICE (320 and 380 mm Hg)[1] (Continued).

Parameter investigated	Experimental condition	Observations
Epithelialization of wound surface	Controls	Wound essentially completely covered with or without normal appearance of mesothelium.
	Hypoxic	Same.
Mast cell population (also see Figures 9 and 10)	Controls (starved)	Absent on day 6 but increasing with time, being located at periphery of wound rather than in granulation tissue.
	Controls (fed)	Same pattern but extent of increase not as pronounced.
	Hypoxic	Same pattern but extent of increase most pronounced.

[1]Unless otherwise specified, controls include animals fed ad libitum and on a limited dietary intake for 14 days, all maintained at 725 mm Hg; both groups of hypoxic animals were lumped together unless otherwise specified.

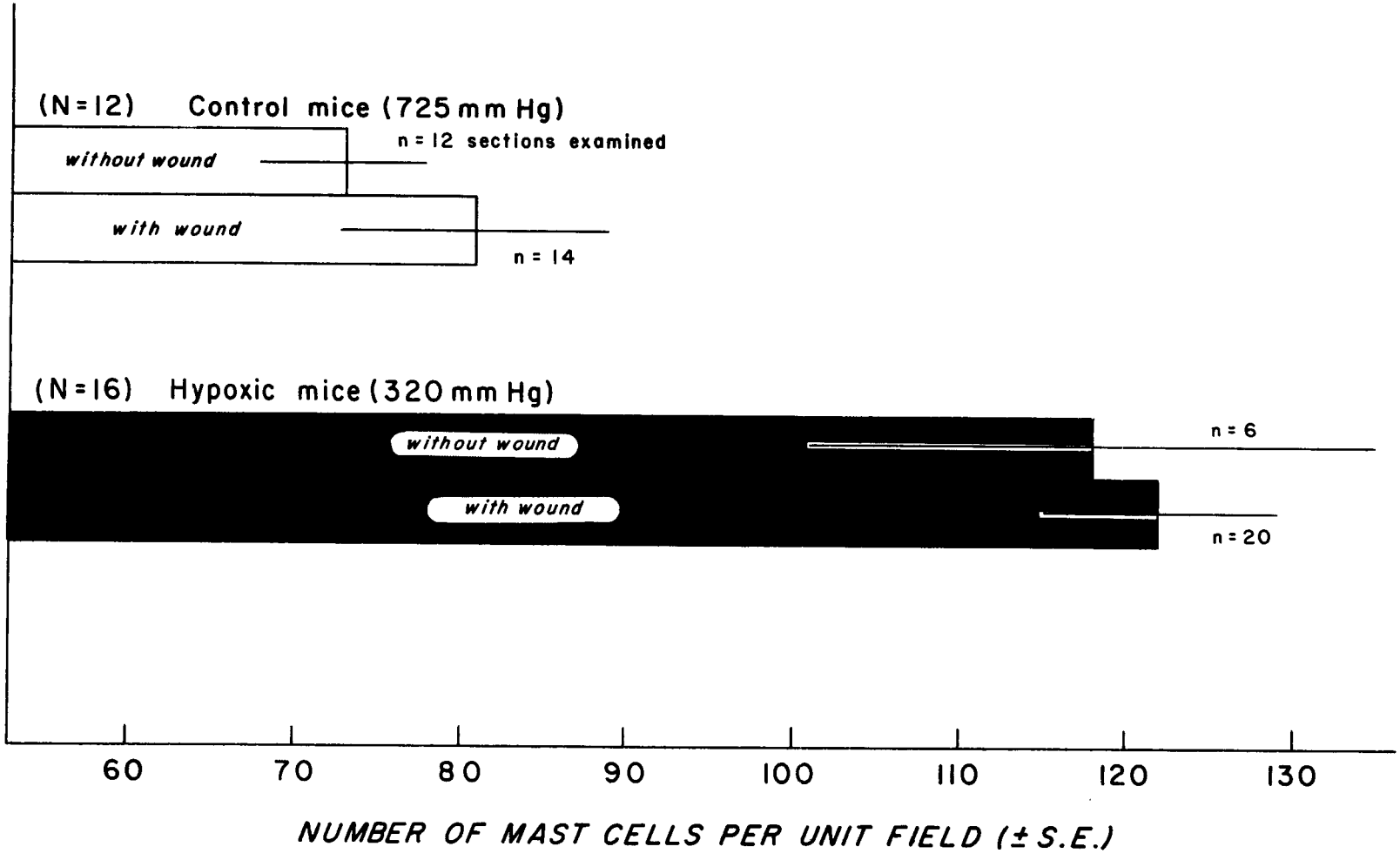

Figure 9. Effect of fundic wounding (serosa and muscularis externa) on mast cell concentration in the fundic submucosa.
(Expressed as mean of counts made six to nineteen days after wounding. Unit field = 8 μ thick section of submucosa (ave. width = 0.44 μ) counted over 10 mm length.)

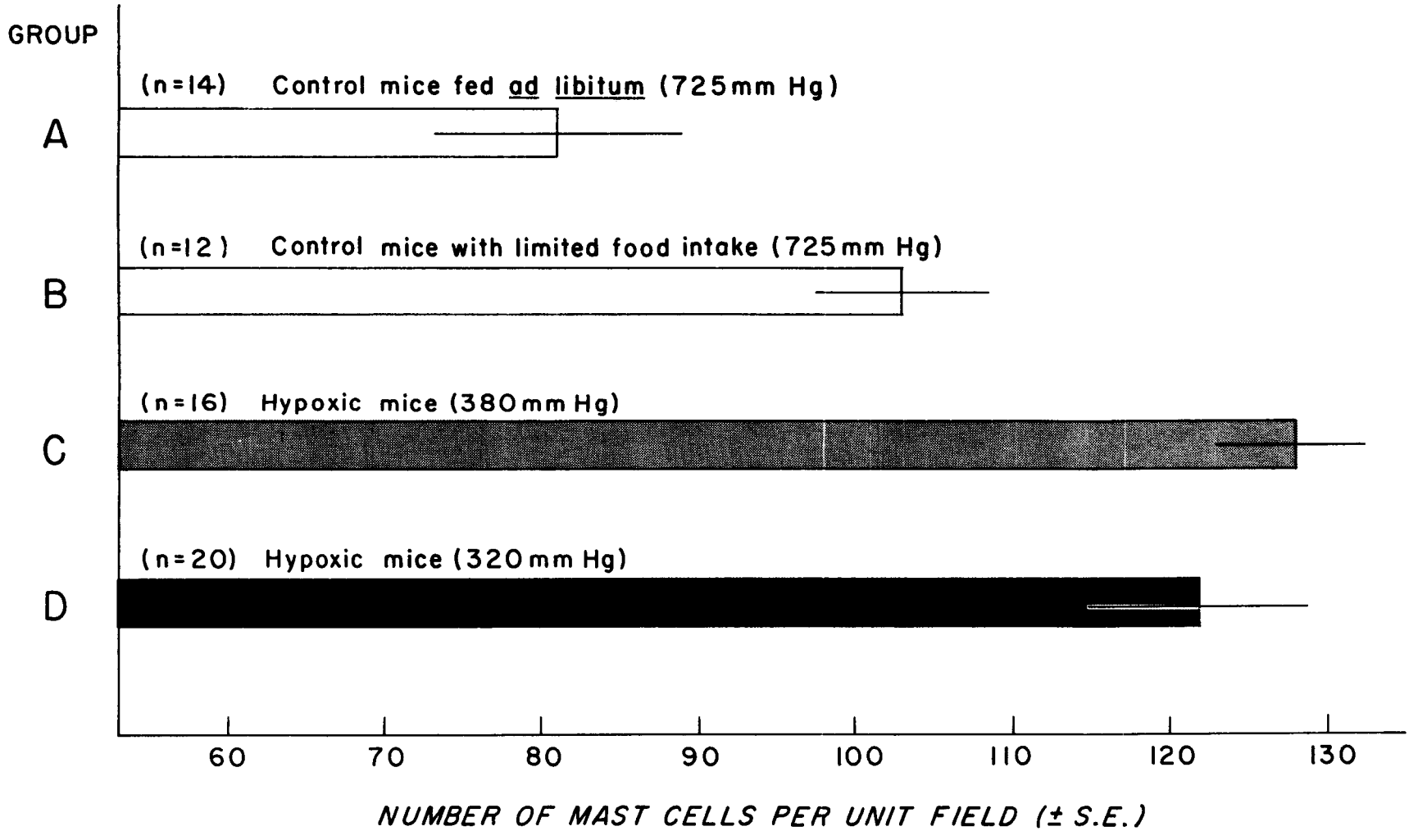

Figure 10. Effect of moderate and severe hypoxia on mast cell concentration in the fundic submucosa of wounded mice.
(Expressed as mean of counts made six to nineteen days after wounding. Unit field = 8 μ thick section of submucosa (ave. width = 0.44 μ) counted over 10 mm length.)

1. Hematocrit response

The first 12 days of hypoxia were characterized by a marked polycythemia (Figure 11). Mean hematocrit in moderately hypoxic animals rose from a normal 42 percent to a maximum value of 65 percent by day 12. Mean hematocrit of severely hypoxic mice was 63 percent by day 12 and 75 percent by day 40.

2. Organ weight changes

Several groups of animals were sacrificed after 21 and 40 days of moderate or severe hypoxia and selected organs were removed and weighed immediately prior to fixation. Mean organ weight data calculated on an actual and relative weight basis are presented in Tables 3 and 4. The major findings were as follows: hypoxia caused adrenal hypertrophy, thymic involution and splenomegaly (Table 3). This was true whether organ weights were calculated on an absolute or relative body weight basis. The magnitude of change was amplified by both duration and severity of exposure. There was a relative increase of heart and testis weights in mice exposed to moderate hypoxia although these organs proved to be slightly smaller than controls on an absolute weight basis (Table 4). With severe hypoxia there was a relative increase in heart weight in 21-day exposed mice and a further increase (both relative and absolute) after 40 days of exposure. Seminal vesicle mass was reduced on both an absolute and relative body weight basis, again directly related to duration and severity of hypoxia.

3. Body weight changes

Growth curves compiled to show the effects of moderate and severe hypoxia on body weight are presented in Figure 12. Hypoxic mice exhibited

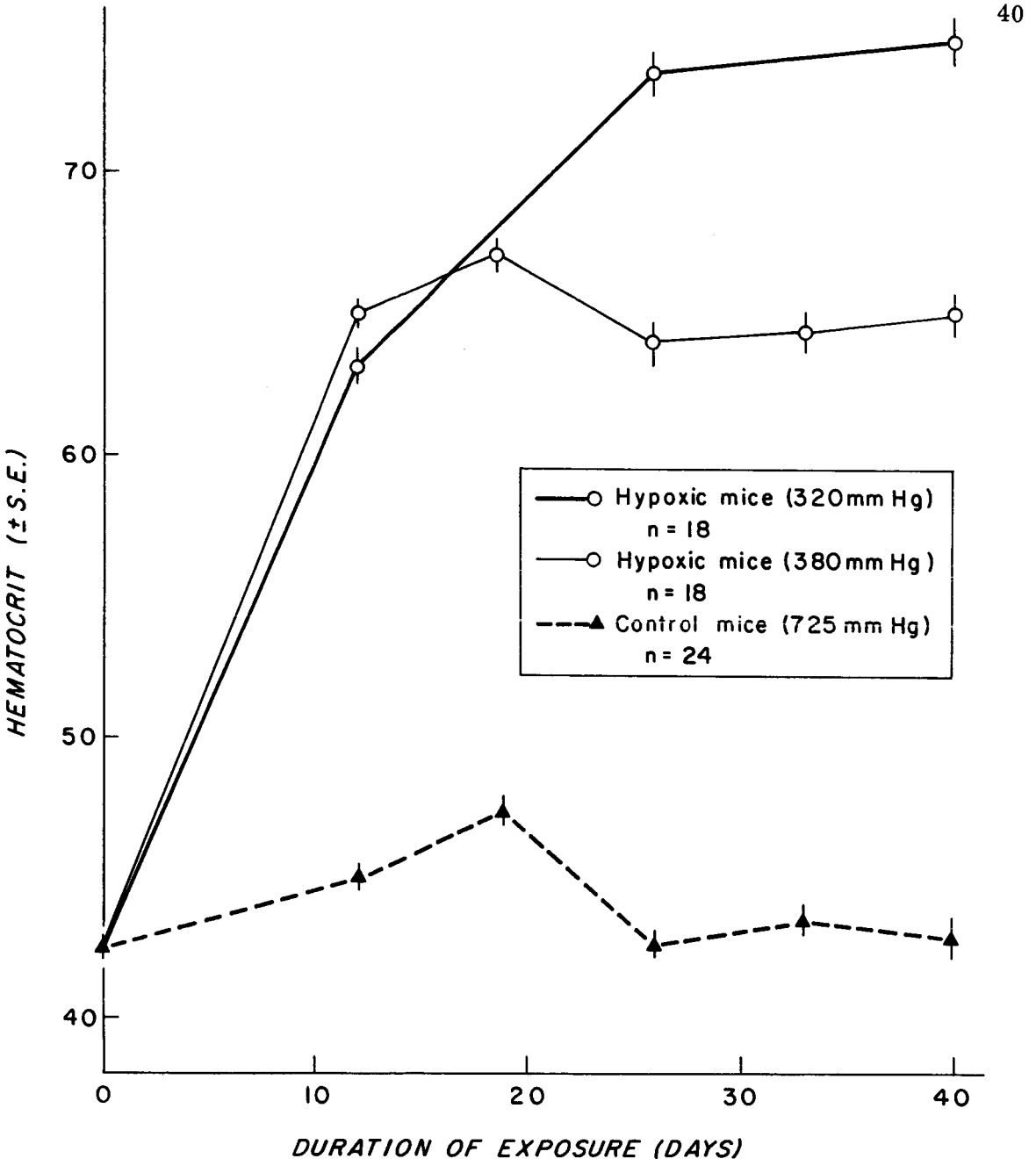

Figure 11. Mean hematocrit response in mice exposed to reduced barometric pressure.

a pronounced weight loss (ca -20 percent) during the first 12 days of exposure. Moderately hypoxic mice gradually gained weight throughout the remaining 28 days of the experiment; severely hypoxic mice maintained their day-12 weight until day 33, after which it rose sharply. The weight of all 40-day exposed hypoxic mice was significantly lower than that of controls.

TABLE 3. WEIGHT ANALYSES OF ADRENALS, THYMUS AND SPLEEN FROM MICE EXPOSED TO REDUCED BAROMETRIC PRESSURE (380 OR 320 mm Hg).[1]

Days of exposure	Number of animals	Barometric pressure	Mean organ weights mgms. ± S. E. Adrenals	Thymus	Spleen
0	21	725 mm Hg	2.5 ± 0.4	25.9 ± 1.2	97.4 ± 3.2
21	12	380 mm Hg	2.9 ± 0.2	16.7 ± 1.6***	102.6 ± 6.1
40	12	380 mm Hg	2.8 ± 0.7	11.5 ± 1.5***	117.8 ± 9.8
21	15	320 mm Hg	3.4 ± 0.1*	2.5 ± 0.2***	129.2 ± 11.6**
40	6	320 mm Hg	3.5 ± 0.3*	2.2 ± 0.5***	203.9 ± 10.5***
			Mean organ weights mgms. /100 gms. body weight ± S. E.		
			Adrenals	Thymus	Spleen
0	21	725 mm Hg	9.1 ± 0.2	93.2 ± 4.7	347.5 ± 9.1
21	12	380 mm Hg	14.8 ± 1.1***	85.0 ± 7.7	528.5 ± 33.6***
40	12	380 mm Hg	14.4 ± 1.0***	59.0 ± 7.5***	613.6 ± 53.3***
21	15	320 mm Hg	23.2 ± 1.1***	16.6 ± 1.3***	860.1 ± 63.8***
40	6	320 mm Hg	16.0 ± 0.9***	10.3 ± 2.2***	934.6 ± 53.8***

[1]Asterisks are used to designate confidence levels of .05 (*), .02(**) and .001 (***), using Student' s t test.

TABLE 4. WEIGHT ANALYSES OF HEART, TESTES AND SEMINAL VESICLES FROM MICE EXPOSED TO REDUCED BAROMETRIC PRESSURE (380 OR 320 mm Hg). [1]

Days of exposure	Number of animals	Barometric pressure	Mean organ weights mgms. ± S. E. Heart	Testes	Seminal vesicles
0	21	725 mm Hg	111.6 ± 2.5	151.7 ± 6.0	150.3 ± 6.9
21	12	380 mm Hg	95.0 ± 3.2***	131.7 ± 3.8**	83.7 ± 5.8***
40	12	380 mm Hg	99.0 ± 2.5**	128.2 ± 5.4**	65.3 ± 4.8***
21	15	320 mm Hg	117.3 ± 4.6	103.5 ± 5.2***	33.5 ± 2.3***
40	6	320 mm Hg	130.2 ± 4.6**	105.1 ± 11.2***	46.5 ± 5.7***
			Mean organ weights mgms. /100 gms. body weight ± S. E.		
			Heart	Testes	Seminal vesicles
0	21	725 mm Hg	399.8 ± 3.4	542.7 ± 17.1	536.8 ± 20.5
21	12	380 mm Hg	487.2 ± 16.0***	672.5 ± 15.8***	426.7 ± 26.4**
40	12	380 mm Hg	514.9 ± 11.2***	669.0 ± 30.4***	334.2 ± 18.6***
21	15	320 mm Hg	790.4 ± 26.4***	694.4 ± 25.2***	237.9 ± 14.0***
40	6	320 mm Hg	536.8 ± 20.5***	471.8 ± 36.5	215.0 ± 23.6***

[1] Asterisks are used to designate confidence levels of .05 (*), .01 (**) and .001 (***), using Student' s t test.

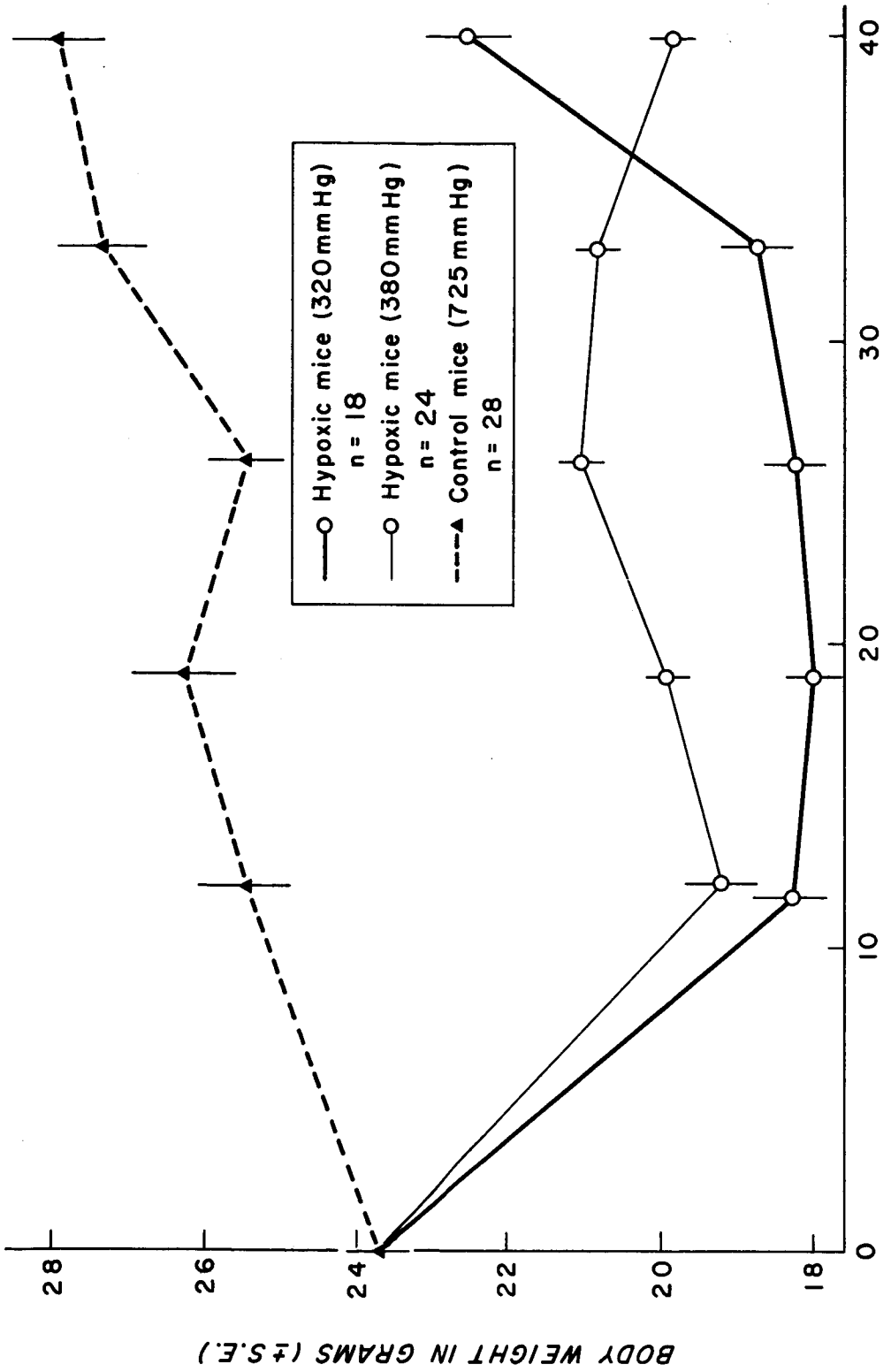

Figure 12. Mean body weight of mice exposed to reduced barometric pressure.

DISCUSSION

Animals exposed to hypoxia exhibit important compensatory cardiovascular changes which increase their capacity to tolerate and eventually adapt to low oxygen. The present study demonstrates the occurrence of adaptively significant peripheral and internal body vascular responses under conditions of reduced barometric pressure. Specifically, exposure to hypoxia triggers an augmented blood flow to internal body tissues while only minimal circulation is maintained in peripheral body regions. In addition, proliferation of new blood vessels is evident in visceral tissues within the first week of hypoxia whereas peripheral angiogenesis is not initiated until the second week of exposure. These results indicate that there is more blood, and therefore more oxygen, in the visceral area than in peripheral tissues during the first days of hypoxia. The conclusion that blood and oxygen adequate for normal tissue functioning are more readily accessible to vital internal organs during this period is supported by observations on wound healing. Fundic stomach repair proceeds normally during acute severe hypoxia whereas closure of external ear punctures is markedly retarded.

Additional insight into the significance of vascular responses to the overall acclimation process can be gained from a consideration of the specific findings in relation to adaptive circulatory responses known to occur during acute and chronic hypoxia.

An early feature of the peripheral vascular response to hypoxia is a marked engorgement of major ear veins. Such dilatation may be brought

about by shunting of oxygenated blood to internal body regions with a resultant decrease in peripheral circulation, inhibition of peripheral venous return to the heart or increased total blood volume. Evidence of a shift in blood flow during acute hypoxia comes from reports of several earlier investigators. Kety and Schmidt (1948) and Lambertsen (1961) have shown an immediate increase in flow through oxygen-dependent brain tissue at the onset of hypoxia; a similar finding has been reported for heart muscle (Eckenhoff et al., 1947; Katz, 1958). On the other hand, vessels of slowly metabolizing skin constrict, resulting in a localized reduction in blood flow volume (Rein et al., 1941). Therefore, it appears that diminished peripheral circulation is a major factor in pinnal venous dilatation. That blood supply is barely adequate for maintenance of ear tissues, but close to minimal requirements, is indicated in this study by sporadic instances of partial desquamation of the ears. Lethargy observed during the first few days of hypoxia also contributes to reduced oxygenation of peripheral tissues since skeletal muscle contraction is known to be a major propulsive force in facilitating return of venous blood to the heart. Since evidence indicates that circulation to ears is diminished and systemic blood volume reduced (Reissman, 1951) during the first week of hypoxia, increased arterial blood volume in the ear is not a factor in venous engorgement. Beneficial consequences of a shift in blood flow during the critical early period of hypoxia are readily apparent if one considers that this would provide vital body organs with the maximum amount of available oxygen.

It is of importance to note that early circulatory alterations resulting in a greater oxygen supply to systemic organs are supplemented by

erythropoiesis and body mass reduction, both of which aid in adjustment to hypoxia. Marked erythropoiesis, which serves to increase the oxygen-carrying capacity of blood (Clark and Otis, 1952), is commonly found during the first two weeks of hypoxia exposure (Van Liere and Stickney, 1963). The concomitant loss of body mass, a manifestation of reduced dietary and water intake during acute hypoxia, lowers the animal' s oxygen requirements (Isenberg, 1966). Although loss of overall body weight occurs, many organs maintain their weight or hypertrophy. For example, adrenal hypertrophy due to stimulation of ACTH secretion, splenomegaly due to more rapid erythropoiesis, and cardiac enlargement are all associated with hypoxia exposure (Van Liere and Stickney, 1963). Maintenance of testis weight may be related to unimpaired male reproductive function at reduced barometric pressures (Van Liere and Stickney, 1963). Thus, a change in circulatory flow pattern is only part of a complex of early adaptive alterations aimed at conserving available oxygen and supplying it primarily to vital body tissues.

An additional means by which oxygen becomes more readily accessible at the cellular level is vascular proliferation. Such hyperplasia is essential to survival and eventual adaptation since it affords a reduction in diffusion distance between blood and cell (Van Liere and Stickney, 1963). As previously observed by Anthony and Kreider (1961), angiogenesis is evidenced in internal body regions within the first few days of hypoxia-exposure. This is in keeping with the concept that a prime purpose of early vascular responses is to supply sufficient oxygen to actively metabolizing tissues. It is evident that adequate oxygenation of internal tissues results from both redistribution of blood to internal body regions and stimulation of new vessel formation.

In contrast to rapid initiation of angiogenesis in internal tissues, vascular proliferation in peripheral body regions is not evidenced until the third week of hypoxia. This retardation is due to tissue hypoxemia resulting from a reduced flow of oxygen-deficient blood through the ear. The hypoxemia, whose severity would be dependent upon barometric pressure, would be sufficient to inhibit synthetic mechanisms essential for vessel growth and proliferation. A relationship between vascular hyperplasia and blood flow has been previously described by Clark and Clark (1939) who studied capillary growth in rabbit ears. Using a "ear chamber" method for in vivo study, they observed rapid capillary proliferation in the presence of normal circulation; angiogenesis was, however, markedly slower in those parts of the ear where circulation was sluggish. It is significant that ear vessel formation is noticeably stimulated when hemal adjustments, e.g. increased hematocrit (Nelson et al., 1963) and greater blood volume (Anthony and Kreider, 1961; Reissman, 1951) have increased the oxygen-carrying capacity of blood (Nelson, Rempe and Anthony, 1963).

In view of the above considerations one would expect the most marked impairment in blood flow and inhibition of vessel formation to occur in peripheral tissues of mice exposed to severe hypoxia. This proved to be the case. Ear veins of severely hypoxic mice are more congested than those of moderately hypoxic animals indicative of a greater reduction in blood flow through these ears. In addition, peripheral vascular proliferation is less extensive in severely than in moderately hypoxic mice during the first three weeks of exposure. In contrast, visceral tissues of both severely and moderately hypoxic mice exhibit equivalent degrees of angio-

genesis, indicating that circulation to internal body tissues is sufficient for vascular hyperplasia in both groups.

Data obtained on wound healing in hypoxic mice provide additional evidence of a shift in blood distribution. Since tissue repair involves anabolic, aerobic processes (Bullough and Laurence, 1957; Houck, 1963), it is intimately dependent upon the available blood supply. Greater blood flow to visceral tissues, at the expense of a lowered circulation to peripheral body regions, during acute hypoxia would therefore explain the observed normal fundic and retarded pinnal healing. Nineteen days of hypoxia acclimation, with its associated compensatory vascular changes leading to increased oxygen availability in peripheral tissues, results in an almost complete restoration of pinnal healing capacity. Indirect support of the above explanation is afforded by studies on food-restricted animals. Although protein depletion may interfere with normal healing processes (DiPasquale and Steinetz, 1964; Rosenberg and Caldwell, 1965), the present study demonstrates that the limited dietary intake associated with acute hypoxia has no effect on repair of ear and fundic wounds.

The conclusion that healing of internal wounds is not altered by hypoxia-exposure is supported by two findings. First, no differences are observed between hypoxic and control mice in the intensity of metachromasia which has been used as an index of healing (Rasmussen, 1966; Thompson, 1966). Second, there is no difference in the gross or microscopic appearance of wound sites which can be attributed to hypoxia-exposure.

An initial absence of mast cells is the wound area, followed by their marked appearance at the wound periphery as healing progresses, has been

reported by other investigators (Michels, 1938; Wichmann, 1955). The functional significance of observed increases in the number of wound and submucosal mast cells of starved and hypoxic mice is not understood. However, available evidence indicates that they have no effect on repair processes. For example, Fiori-Donati and Moltke (1960) and Fisher and Hellstrom (1961) reported normal healing in the rat, as indicated by rate of wound contraction, tensile strength and histological appearance of the wound, in spite of almost total mast cell depletion. They imply that repair is independent of mast cells.

The effect of hypoxia-exposure on repair of visceral organs has not been previously investigated; however, retardation of peripheral healing during acute hypoxia is commonly found (Utinka, 1958; Weihe, 1964). Weihe (1964), on the basis of data obtained from a study of integumentary wound healing, suggests that such impairment is the direct result of a rise in plasma adrenocortical hormones. Since the present study establishes the normal cicatrization of fundic stomach lesions at the same time that ear wound closure is retarded, hormone involvement appears unlikely. Rather, a preferential shunting of blood from peripheral to internal body tissues seems responsible for the early selective retardation of peripheral wound closure.

SUMMARY

This study was designed to investigate peripheral and internal vascular responses to hypoxia and their relation to wound healing. Adult male A/HeJ mice were exposed to moderate (380 mm Hg) or severe (320 mm Hg) hypoxia for 7 to 40 days. The extent of peripheral angiogenesis was determined from weekly stereoscopic drawings of ear vasculatures in hypoxic and control mice; vascular proliferation was also microscopically observed in visceral tissues. Healing of peripheral wounds (ear puncture) was expressed as percentage of puncture filled with granulation tissue; internal body wound healing (electrocautery of fundic stomach) was studied histologically and histochemically (toluidine blue 0). Supplemental information on vascular changes and wound repair was obtained from microscopic analysis of 6-8μ sections. During the course of study, data on hematocrit and body and organ weight changes were also recorded. A total of 234 animals was used.

Major findings were as follows: (1) During the first week of exposure, blood was pooled in visceral tissues while circulation in the ears was reduced. (2) Proliferation of terminal vessels was initiated in the pinna after the first week of hypoxia-exposure; in contrast, vascular hyperplasia was evident in internal body regions within the first week of hypoxia. (3) Cicatrization of ear punctures was retarded under acute severe hypoxia but prior acclimation for 19 days restored the healing potential to almost normal levels; peripheral repair was normal under either acute or chronic moderate hypoxia. (4) Moderate or severe hypoxia had no effect on fundic wound healing as judged by the criteria of histological appearance and metachromasia.

It was concluded that hypoxia-exposure triggers a shift in blood distribution such that internal body tissues receive more blood while peripheral regions are supplied with only minimal amounts; another important effect of hypoxia is the stimulation of angiogenesis, first in the visceral tissues and later in peripheral regions. These vascular responses ensure that oxygen adequate for normal functioning is available primarily to vital organs and is subsequently restored to tissues throughout the body. Adaptive vascular changes were also shown to be intimately related to the retardation of peripheral wound healing often associated with hypoxia.

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APPENDIX

Summary Tables of the Data

TABLE 5. EFFECT OF HYPOXIA ON VENOUS HYPERPLASIA IN THE MOUSE EAR.

	Total veins/mouse ear		
	Controls (725 mm Hg) n = 16[1]	Hypoxic (320 mm Hg) n = 16	Hypoxic (380 mm Hg) n = 6
Days of exposure	Average number of veins ± S. D. (S. E.)	Average number of veins ± S. D. (S. E.)	Average number of veins ± S. D. (S. E.)
0	40. 9 ± 13. 0 (2. 0)		
12	45. 6 ± 2. 9 (0. 7)	54. 6 ± 6. 4 (1. 6)	64. 7 ± 12. 2 (5. 0)*
19	51. 8 ± 5. 6 (1. 4)	73. 6 ± 9. 4 (2. 4)*	96. 0 ± 12. 6 (5. 2)*
26	56. 1 ± 7. 0 (1. 8)	96. 2 ± 11. 7 (2. 9)*	104. 3 ± 13. 4 (5. 5)*
33	60. 7 ± 7. 0 (1. 8)	102. 8 ± 10. 3 (2. 6)*	117. 2 ± 19. 1 (7. 8)*
40	62. 6 ± 7. 4 (1. 9)	110. 7 ± 12. 7 (3. 2)*	122. 0 ± 18. 6 (7. 6)*

[1] n represents the number of ears studied; day 0 counts were based on 20 mouse ears.

*Significant at less than . 05 level of confidence using Student' s t test when compared to corresponding controls.

TABLE 6. EFFECT OF HYPOXIA ON ARTERIAL HYPERPLASIA IN THE MOUSE EAR (380 mm Hg).

	Total arteries/mouse ear	
	Controls (725 mm Hg) n = 3[1]	Hypoxic (380 mm Hg) n = 5
Days of exposure	Average number of arteries ± S. D. (S. E.)	Average number of arteries ± S. D. (S. E.)
0	24.0 ± 5.7 (2.0)	
12	32.0 ± 9.9 (7.1)	32.8 ± 10.4 (4.6)
19	39.0 ± 15.6 (11.1)	52.6 ± 5.3 (2.4)
26	28.0 ± 10.4 (6.0)	55.0 ± 11.6 (5.2)*
33	32.0 ± 2.2 (1.6)	60.8 ± 15.0 (6.7)*
40	26.5 ± 3.5 (2.5)	59.2 ± 14.4 (6.4)*

[1]n represents the number of ears studied; day 0 counts were based on the total 8 ears.

*See Table 5.

TABLE 7. EFFECT OF HYPOXIA ON VENOUS DILATATION IN THE MOUSE EAR (320 mm Hg).

	Total veins dilated/mouse ear	
	Controls (725 mm Hg) n = 16[1]	Hypoxic (320 mm Hg) n = 16
Days of exposure	Average number dilated ± S. D. (S. E.)	Average number dilated ± S. D. (S. E.)
12	0.5 ± 1.0 (0.2)	2.2 ± 2.4 (0.6)*
19	0.8 ± 1.1 (0.3)	5.0 ± 3.2 (0.8)*
26	1.1 ± 1.2 (0.3)	8.4 ± 4.3 (1.1)*
33	0.9 ± 1.0 (0.2)	9.1 ± 4.8 (1.2)*
40	0.9 ± 1.1 (0.3)	9.6 ± 5.2 (1.3)*

[1]n represents the number of ears studied.

*See Table 5.

TABLE 8. EFFECT OF HYPOXIA ON EAR WOUND CLOSURE.[1]

Treatment	Number of animals	Average percent of closure ± S. D. (S. E.)
Wounded day zero of hypoxia exposure (320 mm Hg)	20	62.7 ± 18.2 (4.1)*
Wounded day zero of hypoxia exposure (380 mm Hg)	29	92.9 ± 7.0 (1.3)
Acclimated before wounding and kept at 320 mm Hg	13	88.0 ± 9.2 (2.6)
Acclimated before wounding and kept at 380 mm Hg	17	89.3 ± 10.2 (2.5)
Controls fed ad libitum (725 mm Hg)	21	94.5 ± 8.2 (1.8)
Controls with limited food intake (725 mm Hg)	5	91.7 ± 1.6 (0.7)

[1]Expressed as percentage of puncture filled with granulation tissue 21 days after wound induction.

*See Table 5.

TABLE 9. EFFECT OF HYPOXIA ON VENOUS HYPERPLASIA IN THE EAR WOUND AREA.

	Total veins/ear wound[1]	
	Controls (725 mm Hg) n = 8[2]	Hypoxic (320 mm Hg)[3] n = 8
Days after wounding	Average number of veins ± S. D. (S. E.)	Average number of veins ± S. D. (S. E.)
7	22.1 ± 5.7 (2.0)	27.0 ± 5.4 (1.9)
14	36.0 ± 11.6 (4.1)	35.6 ± 7.4 (2.6)
21	29.4 ± 7.1 (2.5)	37.6 ± 7.4 (2.6)*

[1]Total calculated on an initial base value of 7 veins in the area prior to wound induction.

[2]n represents the number of wounds studied.

[3]Hypoxic mice were acclimated 19 days prior to receiving wound.

*See Table 5.

TABLE 10. MAST CELL NUMBERS IN THE FUNDIC SUBMUCOSA AS AFFECTED BY WOUNDING, STARVATION AND HYPOXIA.[1]

Treatment	Average number mast cells/unit field[2]		
	N[3]	n	Average number of cells ± S. D. (S. E.)
Controls; fed ad libitum; without wound	3	12	7.3 ± 1.7 (0.5)
Controls; fed ad libitum; with wound	12	14	8.1 ± 3.0 (0.8)
Controls; limited food intake; with wound	6	12	11.3 ± 1.9 (0.6)*
Hypoxic (320 mm Hg); without wound	6	6	11.8 ± 5.3 (2.2)*
Hypoxic (320 mm Hg); with wound	11	20	12.2 ± 3.2 (0.7)*
Hypoxic (380 mm Hg); with wound	9	16	12.8 ± 1.8 (0.4)*

[1]Expressed as mean of counts made 6 to 19 days after onset of the experimental period.

[2]Unit field = 8 μ thick section of submucosa (average width = 0.44 μ) counted over 10 mm length.

[3]N represents number of animals; n represents the number of tissue sections examined.

*Significant at less than .05 level of confidence using Student' s t test. In all instances comparisons were made with the two control groups fed ad libitum.

TABLE 11. MEAN HEMATOCRIT OF MICE EXPOSED TO REDUCED BAROMETRIC PRESSURE.

	Average hematocrit					
	Control mice (725 mm Hg)		Hypoxic mice (320 mm Hg)		Hypoxic mice (380 mm Hg)	
Days of exposure	No. of mice	$\bar{X}$ ± S. D. (S. E.)	No. of mice	$\bar{X}$ ± S. D. (S. E.)	No. of mice	$\bar{X}$ ± S. D. (S. E.)
0[1]	59	42. 5 ± 3. 2 (0. 4)				
12	23	44. 9 ± 2. 4 (0. 5)	18	63. 2 ± 3. 2 (0. 8)*	18	65. 1 ± 2. 1 (0. 5)*
19	6	47. 5 ± 1. 0 (0. 4)	--	--	16	67. 1 ± 1. 8 (0. 4)*
26	21	42. 7 ± 2. 7 (0. 6)	17	73. 6 ± 3. 0 (0. 7)*	17	64. 1 ± 2. 4 (0. 6)*
33	4	43. 5 ± 1. 3 (0. 6)	--	--	16	64. 4 ± 2. 3 (0. 6)*
40	17	42. 9 ± 3. 2 (0. 8)	16	74. 8 ± 3. 6 (0. 9)*	17	65. 2 ± 2. 1 (0. 5)*

[1]Based on initial hematocrits of all animals used in this study.

*See Table 5.

TABLE 12. MEAN BODY WEIGHT OF MICE EXPOSED TO REDUCED BAROMETRIC PRESSURE.

	Average body weight					
	Control mice (725 mm Hg)		Hypoxic mice (320 mm Hg)		Hypoxic mice (380 mm Hg)	
Days of exposure	No. of mice	$\overline{X}$ ± S. D. (S. E.)	No. of mice	$\overline{X}$ ± S. D. (S. E.)	No. of mice	$\overline{X}$ ± S. D. (S. E.)
0[1]	64	23.7 ± 2.9 (0.4)				
12	24	25.5 ± 2.9 (0.6)	18	18.3 ± 2.1 (0.5)*	18	19.2 ± 2.2 (0.5)*
19	22	26.3 ± 3.3 (0.7)	17	18.0 ± 1.8 (0.4)*	24	19.9 ± 1.6 (0.3)*
26	22	25.5 ± 2.5 (0.5)	17	18.3 ± 1.6 (0.4)*	22	21.0 ± 1.5 (0.3)*
33	19	27.4 ± 2.8 (0.6)	17	18.7 ± 2.1 (0.5)*	17	20.8 ± 1.1 (0.3)*
40	21	27.9 ± 3.0 (0.6)	16	22.5 ± 2.4 (0.6)*	22	19.8 ± 1.4 (0.3)*

[1]Based on initial body weights of all groups used in this study.

*See Table 5.