

LAUNCH CONDITIONS MIGHT AFFECT THE FORMATION OF BLOOD VESSELS IN THE QUAIL CHORIOALLANTOIC MEMBRANE

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SUMMARY

As a part of the first joint USA—Russian MIR/Shuttle program, fertilized quail eggs were flown on the MIR 18 mission. Post-flight examination indicated impaired survival of both the embryos in space and also of control embryos exposed to vibrational and *g*-forces simulating the conditions experienced during the launch of Progress 227. We hypothesized that excess mechanical forces and/or other conditions during the launch might cause abnormal development of the blood supply in the chorioallantoic membrane (CAM) leading to the impaired survival of the embryos. The CAM, a highly vascularized extraembryonic organ, provides for the oxygen exchange across the egg shell and is thus pivotal for proper embryonic development. To test our hypothesis, we compared angiogenesis in CAMs of eggs which were either exposed to the vibration and *g*-force profile simulating the conditions at launch of Progress 227 (synchronous controls), or kept under routine conditions in a laboratory incubator (laboratory controls). At various time points during incubation, the eggs were fixed in paraformaldehyde for subsequent dissection. At the time of dissection, the CAM was carefully lifted from the egg shell and examined as whole mounts by bright-field and fluorescent microscopy. The development of the vasculature (angiogenesis) was assessed from the density of blood vessels per viewing field and evaluated by computer aided image analysis. We observed a significant decrease in blood-vessel density in the synchronous controls versus "normal" laboratory controls beginning from day 10 of incubation. The decrease in vascular density was restricted to the smallest vessels only, suggesting that conditions during

the launch and/or during the subsequent incubation of the eggs may affect the normal progress of angiogenesis in the CAM. Abnormal angiogenesis in the CAM might contribute to the impaired survival of the embryos observed in synchronous controls as well as in space.

Key words: quail (*Coturnix coturnix japonica*); chorioallantoic membrane (CAM); angiogenesis; morphometry; image analysis; space flight; microgravity

INTRODUCTION

As in every other biological process on Earth, embryogenesis occurs under the influence of gravity. Indeed, gravitational forces seem to play a major role in the initial stages of embryonic development, such as proper positioning of the embryo, separation of yolk from albumen, etc. (Suda *et al.*, 1994). Previous studies have indicated that exposure to weightlessness (microgravity) during space flight might result in abnormalities in embryonic and fetal development in birds, amphibians and mammals, especially when eggs/embryos are sent into space immediately after fertilization (Suda *et al.*, 1994). Such abnormalities include early embryonic death (Hullinger, 1993) as well as delayed development (Guryeva *et al.*, 1993) and alterations in normal positioning of horizontal cleavage furrow, blastocoel and dorsal lip (Neff *et al.*, 1993). For example, the embryonic development of chickens was halted in the early stages, if the eggs were exposed to microgravity immediately or shortly (2 days) after fertilization. By contrast, embryos exposed to microgravity after 7—10 days of normal gravitational conditions developed

normally (S u d a *et al.*, 1994). Based on these observations, we hypothesized that in addition to microgravity, other parameters associated with space flight (such as excessive forces during the launch, and/or the incubation conditions aboard the space vehicles) might also affect embryonic development by altering the development of the vasculature during early stages of embryogenesis.

Vascular development can occur through two fundamentally different processes, termed vasculogenesis and angiogenesis (R i s a u *et al.*, 1988; P o o l e and C o f f i n, 1989; R i s a u, 1991). Vasculogenesis describes the development of the earliest (extraembryonic) blood vessels from local precursor cells. By contrast, angiogenesis depicts the complex process of subsequent formation of microvessels by sprouting from the existing larger ones. The effects of microgravity on angiogenesis are not known, but some indications in the literature suggest that microgravity during space flight might impair angiogenesis (S u d a *et al.*, 1994).

The chorioallantoic membrane (CAM) is a highly vascularized extraembryonic membrane, which in avians provides for gas exchange with the outside environment and the transport of minerals (calcium) from the egg shell to the embryo. The CAM originates from a fusion product of the mesodermal layer of the chorion and the mesodermal layer of the allantois; it then attaches itself to the inner wall of the egg shell (G i l b e r t, 1995). Studies of the CAM in the chicken (less in the quail) have proved a useful tool in understanding fundamental processes involved in angiogenesis (D e F o u w *et al.*, 1989; A u s p r u n k *et al.*, 1991; K u r z *et al.*, 1995; P a p a d i m i t r i o u *et al.*, 1993; P a t a n *et al.*, 1993; W i l t i n g *et al.*, 1993; I r u e l a - A r i s p e *et al.*, 1995).

In this study, we tested our hypothesis by evaluating vascular development in CAMs of quails eggs exposed to vibrational and *g*-forces simulating the launch of the Russian space craft Progress 227 and subsequently incubated under conditions equivalent to those aboard the MIR 18 mission (synchronous controls). Our results indicate a sharp decrease in the microvascular density in the synchronous controls as compared to normal laboratory controls.

MATERIALS AND METHODS

Quail-eggs

This study is a part of the ongoing USA—Russian collaborative space research program carried out during joint Space Shuttle/MIR mission. As in previous studies on the effects of space flight on avian development in eggs from a hypodynamic strain of Japanese quails (*Coturnix coturnix japonica*) were used (B o d a *et al.*, 1992). The eggs used for the ground controls and for the synchronous controls were from a bird colony maintained at the facilities of the Institute for Biomedical Problems (IBMP) in Moscow. Ground controls and synchronous controls (48 each) were from the same batch of eggs. As previously described, synchronous control eggs were exposed to the same vibrational/gravitational profile as the "space" eggs (G u r y e v a *et al.*, 1993). In this particular

study, the synchronous control eggs were exposed to mechanical factors simulating the launch profile of Progress 227: Linear acceleration — 6 *g* for 10 min, impact acceleration — 5 x 40 *g* for 1—5 min. In addition, the cells were also exposed for 10 min to a complex spectrum of vibrational acceleration. Subsequently, the eggs were incubated under identical conditions and in the same kind of incubator as the one aboard MIR 18.

The eggs used for the "normal" laboratory controls were from a colony of hypodynamic quails maintained by the Poultry Management Facilities at Purdue University, Lafayette, IN. Fertilized eggs were shipped per courier mail at ambient temperature. Upon arrival in the laboratory in Milwaukee, the eggs were incubated at 37 °C at saturating humidity for 16 days. The eggs were rotated manually 3 times per day.

Fertility for all groups (laboratory controls in Milwaukee, ground controls and synchronous controls in Moscow) was equally high (> 95 %). Embryonic development (up to the time point of fixation) in both the ground and the laboratory control groups was indistinguishable. More than 90 % of all fertilized eggs developed to hatch (i.e. approximately up to the time of fixation), as assessed by staging according to Hamburger and Hamilton (S a n c h e s, 1992). However, there was significantly more premature arrest of embryonic development in the synchronous controls, especially at later stages of incubation. At all stages, evaluation of the vasculature was carried out only on those CAMs, where the embryos appeared fully developed.

Tissue fixation

For each time point (embryonic days 7, 10, 12, 14, and 16) the eggs (8 each per group, in the studies conducted in Moscow, 7 in the studies done in Milwaukee) were cracked and the air sac was punctured. To fix the ground and the synchronous controls (in Moscow), two cracked eggs each were placed into specially designed storage bags containing 75 ml 4 % buffered paraformaldehyde. To fix the eggs from the "normal" laboratory control group in Milwaukee, two to three eggs each were placed in 100 ml jars filled with 10 % buffered formalin solution (Fisher Scientific). The fixed eggs were stored at 4 °C in fixative for approximately 28 days and subsequently stored at room temperature for up to two months in phosphate buffered saline (PBS) until dissection. The fixation and storage conditions were similar to those aboard MIR 18.

Light microscopic and quantitative evaluation by computer-aided analysis

Following fixation, ground and synchronous control eggs were dissected at NASA AMES Research Center. The eggs were opened longitudinally and the embryos carefully removed. The CAM, closely adhering to both halves of the egg shell, was carefully removed and placed in diethylpyrocarbonate-treated, RNase free PBS. The specimens were then transported to Milwaukee for further examination. All laboratory controls were handled in Milwaukee as described above. To facilitate dissections, the CAMs were spread out in a PBS-filled Petri dish. For each CAM, several specimens, approximately 2 x 2 cm, were placed flat on a microscope slide with a drop of PBS, covered with a cover slip and mounted on the stage of

A Nikon Microphot-F photo microscope. Two visualization methods were employed: for bright light microscopy, a plan 2.5 x long working distance objective was used. Autofluorescence, which particularly highlights the blood vessels in formaldehyde or formalin fixed samples (L e l k e s *et al.*, 1994) was visualized using a Nikon plan 4 x (0.1 NA) objective. For each CAM we evaluated several, random field areas (approximately 2.4 mm²). In some instances, after contrast enhancement the images were directly digitized using a Sony CCD-IRIS/RGB color video camera. Alternatively, we first prepared color slides (using a Nikon F-35WA camera and Kodak Ektachrome 200 ASA color reversal film) and then digitized the images on the slides. All images were captured and analyzed using PC-based software (ImagePro-Plus Media Cybernetics). For the laboratory controls and the ground controls, images from 7 samples per fixation day, for the synchronous controls images from 4 samples were evaluated. Parameters analyzed included the number of vessels per viewing field and the diameter of each vessel (in mm). Using MathCAD software, the numerical data were subsequently classified according to the diameters (d) into small ($0 < d \leq 75 \mu\text{m}$), medium $75 < d \leq 350 \mu\text{m}$, and large vessels ($d > 350 \mu\text{m}$). All numerical values are given as means \pm standard deviations. The statistical differences between the means were assessed using ANOVA analysis (InStat software) and considered to be significant for $p < 0.05$.

RESULTS AND DISCUSSION

The chorioallantoic membrane (CAM) is a vital extra-embryonic organ that provides for adequate gas exchange across the egg shell and facilitates the transfer of minerals in particular calcium, from the egg shell to the developing embryo (G i l b e r t, 1995). Therefore, the appropriate development of the vasculature in the chorioallantoic membrane is pivotal for the proper development of the embryo. Angiogenesis in the avian CAM has been studied extensively specifically in chick, less in quails (K u r t z *et al.*, 1995; P a p a d i m i t r i o u *et al.*, 1993; W i l t i n g *et al.*, 1992; M i s s i r l i s, 1990).

Shown in Fig. 1 is a partial view of the highly vascularized chorioallantoic membrane (CAM) in a quail egg 16 days after fertilization. Upon longitudinal dissection of the egg and removal of embryo, the CAM is clearly discernible in close apposition to the egg shell. The organized, fern-like network of the prominent large vessels is evident even at this low magnification. The composite micrograph in Fig. 2 depicts the gradual development of the microvasculature in ground control quails. In this picture the autofluorescence of the formaldehyde-fixed tissues (L e l k e s *et al.*, 1994) was used to visualize details of the evolving vasculature. On embryonic day 7 some of the larger vessels in the CAM were well developed, while only very few arborizing microvessels were visible. The number of these microvessels increased strongly over the period of embryonic and fetal quail development. Of particular



Fig. 1. Visualization of a part of the chorioallantoic membrane inside a "normal" quail egg shell, 17 days after fertilization. Note the clearly visible pattern of (dark) blood vessels. Digitized image of a black and white video still photography, obtained with a microNIKKOR lens ($f = 55 \text{ mm}$; $1 : 2.8$) attached to a mtI DAGE Series 68 video camera

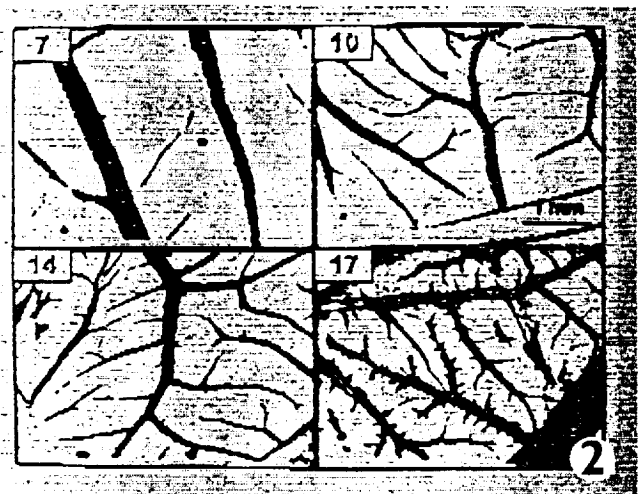


Fig. 2. Angiogenesis in the CAM during normal quail embryonic development: This composite picture, prepared after digitization of individual fluorescent images, depicts the normal development of the vascular architecture in the quail CAM on days 7, 10, 14 and 17. Digitized images of whole mounts of CAMs were obtained as described in Materials and Methods. Note the gradual increase in the number of small vessels over time. Original magnification 50 x

interest is the regular appearance of microvascular branching, which is similar to that observed in chick CAMs (D e F o u w *et al.*, 1989; K u r z *et al.*, 1995; S a n d a u and K u r z, 1994). In contrast to the laboratory controls, the regular pattern of vascular development in CAMs retrieved from the synchronous control eggs appeared to be altered (Fig. 3). There seemed to be fewer vessels per viewing field.

In order to quantitate our observations we used computer-aided image analysis. We first assessed the time course and extent of blood vessel development

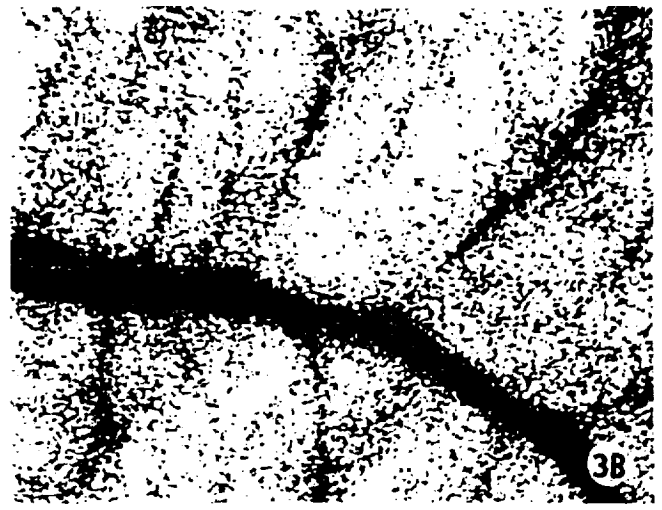


Fig. 3. Comparison of blood vessels architecture between laboratory controls and synchronous controls. Note the regular, arborizing pattern of the vascular architecture of the normal controls (Fig. 3A) of a 16 days old CAM. The synchronous controls (Fig. 3B) contain conspicuously fewer vessels. Original magnification 37.5 x

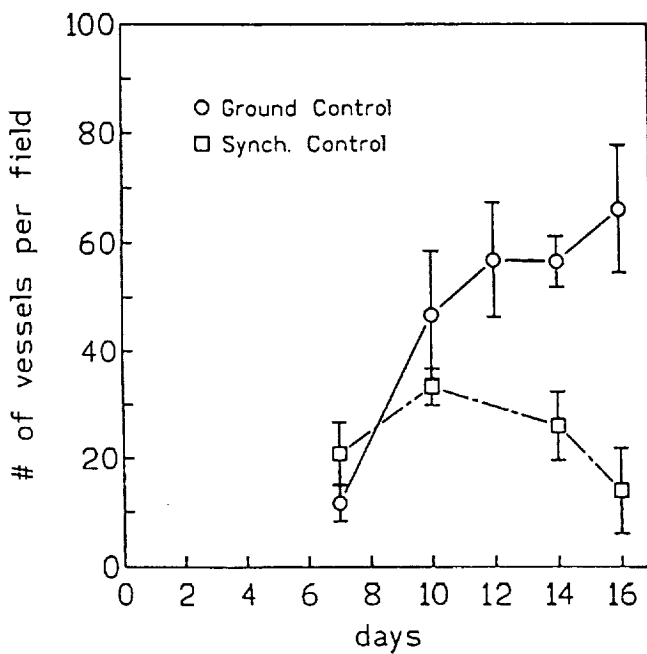


Fig. 4. Total blood vessel count in the developing quail CAM. In normal controls, the total number of blood vessels plateaus approximately after day 12. By contrast, in synchronous controls, the total blood vessel count, which is initially indistinguishable from that in controls, decreases significantly at later stages of embryonic development. The number of blood vessels per viewing field was analyzed by computer aided image analysis, as detailed in Materials and Methods

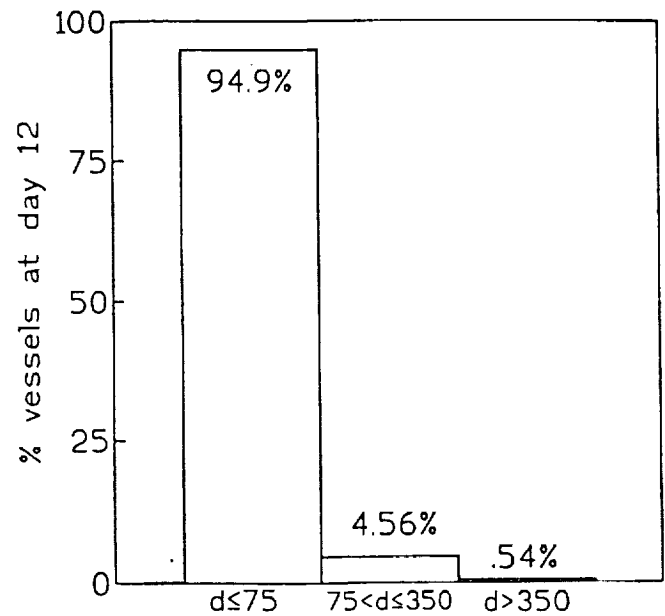


Fig. 5. Frequency distribution of the different vessel sizes in "normal" control quail CAMs on day 12 of embryonic development. The histogram is classified according to the various, arbitrarily chosen size ranges and indicates the predominance of small, capillary-sized vessels in the late stages of normal development. The size of the vessels was assessed by computer aided image analysis, as detailed in Materials and Methods

(angiogenesis) in "normal" laboratory controls and then compared these data to the time course and extent of angiogenesis in ground controls and in synchronous controls, which simulated all flight parameters (vibration, acceleration, deceleration, etc.) of the launch conditions of Progress 227.

As seen in Fig. 4, the number of blood vessels in the normal laboratory controls sharply increases between

embryonic days 7 and 10. Beyond day 12 the number of blood vessels remains practically constant. Similar data were observed for the ground controls at IBMP (data not shown). A very similar pattern of angiogenesis was also reported for the development of the vasculature in chick CAMs, albeit with a slightly different time course (Kurz *et al.*, 1995; Papadimitriou *et al.*, 1993).

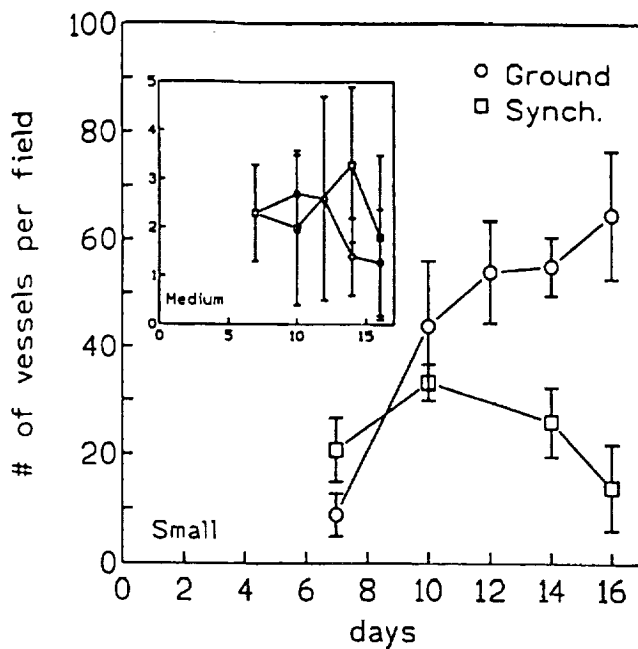


Fig. 6. Launch simulation and/or subsequent incubation conditions specifically affect the development of angiogenesis, as assessed by the decline in the number of small (capillary size) blood vessels in the synchronous control. By contrast, there is no statistically significant difference in the number of medium sized vessels between synchronous and ground controls

Quantitative morphometry confirmed the visual impression of reduced angiogenesis in synchronous controls: As seen in Fig. 4, early on in CAM development, the number of blood vessels per viewing field in the synchronous controls was similar in the normal controls, if not somewhat higher. However, starting from embryonic day 10, the total number of blood vessels decreased sharply. For example, as exemplified in Tab. 1, on embryonic day 16, there were 14 ± 8 blood vessels in the synchronous controls as compared to 66 ± 16 in the laboratory controls or 45 ± 15 in the ground controls.

In further analyzing our morphometric data, we categorized the blood vessels according to their size distribution. The histogram of blood vessel size distribution in the "normal" lab. controls; e.g. on day 12 (Fig. 5) indicates that the majority of all vessels were microvessels in the range between 0 and 75 micrometers in diameter: approximately 95 % of all the blood vessels at this stage are in the range of the smaller vessels ($d \leq 75$ mm), about 4.6 % in an intermediate range ($75 < d \leq 350$ mm), while less than 0.5 % of the vessels are larger than 350 mm.

When the data presented in Fig. 4 were re-analyzed according to the size distribution shown in Fig. 5, we observed that the decline in vessels numbers in the synchronous controls is caused by the gradual disappearance of the smallest vessels between 0—75 micrometers (Fig. 6). By contrast, no statistical significance is observed in the number of the medium size vessels (between 75 and 350 mm) which remains

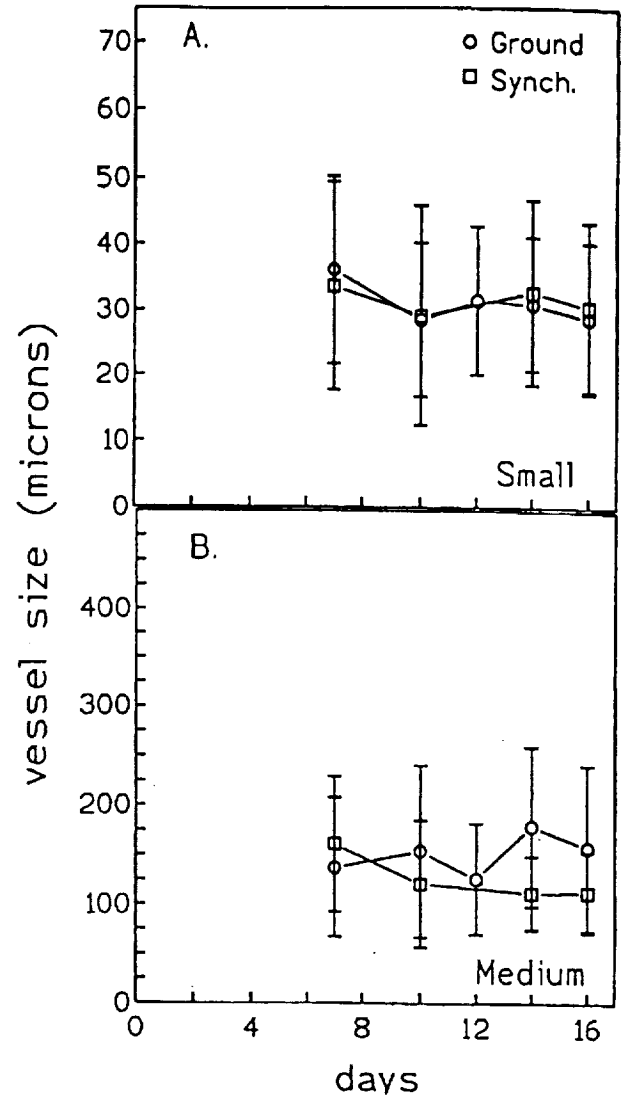


Fig. 7. There is no effect of launch or incubation conditions on the average width of the existing individual blood vessels in the different populations. For both the small and medium sized vessels, there is no statistical difference in the vessel diameters

constant throughout entire incubation period (Fig. 6, insert).

Quantitative morphometry was used to measure the width of individual blood vessels. Throughout the entire incubation period, the average width of individual vessels within the different size groups was not altered (Fig. 7). When averaged over the entire incubation time, the mean vessels diameters of the smallest vessels were 31.1 ± 5.3 mm and 31.5 ± 7.5 mm for laboratory and synchronous controls, respectively. For the medium sized vessels the mean diameters were 125 ± 32.3 mm and 126 ± 27.2 mm, respectively. These findings suggest that once these vessels are formed, the physical constitution of these vessels, as assessed by their width, is not further affected.

Our results indicate a startling detrimental effect of the "launch" conditions for this particular experiment and/or incubations on the development of the chorio-allantoic membrane in the synchronous controls. This

deleterious effect is manifested visibly in a sharp reduction in the number of microvessels. Our results suggest that vibrational or gravitational forces experienced at launch significantly mar angiogenesis in the quail CAM. On the other hand, an adverse effect of the incubation conditions in the "synchronous" incubator cannot be fully excluded. Detailed analysis of the morphometric data clearly points to a selective impairment of some of the processes leading to angiogenesis, i.e. the *de novo* formation of the new, small capillaries (Figs. 4 and 6). Previous studies on angiogenesis have clearly shown that the angiogenic process is a multifaceted process, which involves endothelial cell migration and proliferation, as well as the formation and remodelling of the extracellular matrix. Angiogenic growth factors (Kurz *et al.*, 1995) as well as mechanical forces have been implicated as primary driving forces for the angiogenic process (Hudlicka, 1992; Hudlicka and Brown, 1993). At present, it is unknown which and how angiogenic factors might be affected by the extreme conditions of gravitational or vibrational forces during launch and/or divergence from normal conditions during the subsequent incubation in the synchronous controls. These findings obviously need further confirmation and, if confirmed, warrant detailed analysis in future studies.

Our results are in line with and extend previous observations of minimal hatchability/development of fertilized chick and quail eggs, that were sent into space at various stages of fertilization (Suda *et al.*, 1994; Hullinger, 1993; Guryeva *et al.*, 1993; Bođa *et al.*, 1992). Eggs that were sent on day 0 or 2 (i.e. immediately after fertilization), did not develop in space, whereas, embryos fertilized and then incubated for 7 or 10 days on the ground prior to launch essentially all developed normally. These results strongly suggest that there is a window during embryonic development which is exquisitely sensitive to gravitation. Indeed, previous studies suggest that gravitational forces might strongly affect the polarity, the organization of the blastoderm and subsequent development of the embryo (Neff *et al.*, 1993). Our studies extend those observations indicating that not only the microgravity conditions, but also some of the mechanical forces experienced during the launch and/or the incubation conditions might contribute to the impaired development of the quail embryos. Based on our results, we hypothesize that some of the factors/parameters which precede/accompany the peak of the angiogenic processes and lead to the rapid formation of microvessels around embryonic day 10 may be affected by the conditions experienced by the egg incubated in the synchronous control incubators. Alternatively, on or around embryonic day 10, the same experimental conditions might initiate some enzymatic/proteolytic processes which might cause angioregression, as assessed by the actual decline in the number of microvessels on days 14 and 16 below the levels found on days 7 and 10 (Fig. 4). During normal

avian embryogenesis the occurrence of angioregression and the stimulation of proteolytic and/or matrix degrading enzymes has been observed albeit only very late in the development of the chick CAM (Iruela-Arispe *et al.*, 1995).

Our results seem to confirm our hypothesis that avian embryos exposed to space flight immediately after fertilization might attribute their developmental abnormalities to the inadequate vascularization and angiogenic development in the chorioallantoic membrane. These results might be important in assessing the effects of space flight on mammalian placental/fetal development in early stages of pregnancy.

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В рамках части первой совместной космической программы США и России, на борту космической станции МИР-18 полетели в Космос оплодотворенные яйца перепела. Обследования после приземления на Земле показывали пониженное переживание эмбрионов в полётном эксперименте и эмбрионов синхронного контроля, которые подвергались вибрационным и гравитационным силам, имитирующим условия во время запуска ПРОГРЕССа-227. Предполагается, что чрезвычайная перегрузка или также другие факторы во время взлета могли стать причиной аномального развития снабжения кровью в хориоаллантоической мембране, что может привести к понижению переживания эмбрионов. Хориоаллантоическая мембрана (очень васкуляризованный экстраэмбриональный орган), который делает возможным обмен кислородом через яичную скорлупу, очень важен для правильного развития эмбриона. Чтобы проверить нашу гипотезу, мы сравнивали ангиогенез в хориоаллантоической мембране яиц, которые подвергались вибрационному и гравитационному воздействию, имитирующему условия во время запуска ПРОГРЕССа-227 (синхронный контроль), или находились в рутинных условиях в лабораторном инкубаторе (лабораторный контроль). В разных промежутках времени во время инкубации яйца фиксировались в параформальдегиде. Хориоаллантоическая мембрана тщательно собиралась с яичной скорлупины и подвергалась обследованию как общий микроскопический препарат с помощью оптического и флюоресцентного микроскопа. Ангиогенез определялся из плотности кровеносных сосудов на одно поле зрения и оценивался с помощью компьютерного анализа образа. Наблюдали знаменательное понижение плотности кровеносных сосудов в синхронном контроле по сравнению с лабораторным контролем, начиная с 10-ого дня инкубации. Понижение плотности кровеносных сосудов ограничивалось только на самые тонкие сосуды, что предполагает, что условия во время взлета и последующей ему инкубации яиц могут повредить нормальный процесс ангиогенеза в хориоаллантоической мембране. Аномальный ангиогенез в хориоаллантоической мембране мог способствовать пониженному переживанию эмбрионов, подвергаемых исследованию в синхронной группе и в полетном эксперименте.