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NASA NAG 2-914 "Effects of Space Travel on Skeletal Myofibers"

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Six Month Report on Tissue Cultured Avian Skeletal Myofibers in the STL/A Module Aboard STS-77.

Summary

Space travel is know to effect skeletal muscle, causing rapid and pronounced atrophy in humans and animals, even when strenuous exercise is used as a countermeasure. The cellular and molecular bases of this atrophy are unknown. Space travel may cause muscle atrophy by a direct effect on the muscle fibers and/or indirectly by reducing circulating levels of growth factors such as growth hormone. The recent development of a tissue culture incubator system for Shuttle Middeck basic science experiments [Space Tissue Loss (STL) Module] by the Walter Reed Army Institute of Research (WRAIR) allows the study of the effects of space travel directly on isolated skeletal myofibers. Avian bioartificial skeletal muscle "organoids" containing differentiated skeletal myofibers and connective tissue fibroblasts were flown aboard the Space Shuttle (Space Transportation System, STS) on Flight STS77, a repeat of a similar experiment flown on STS66. **The results from these two flight experiments show for the first time that space travel has a direct effect on skeletal muscle cells separate from any systemic effects resulting from altered circulating growth factors.**

STS77 flight assays for cellular metabolism, total protein synthesis and protein degradation rates have been completed. The effects of reloading the muscle cells post-flight were also completed by measuring total protein synthesis rates and myosin heavy chain, collagen, and fibronectin accumulation and synthesis following return to Earth.

The rates of cellular metabolism and total protein degradation were <u>not</u> altered during launch (L), indicating <u>no launch-induced cellular stress injury to the muscle cells</u>, a tentative hypothesis made from limited STS66 data.[1] Once in space, the metabolic and protein degradation rates continued to be similar to ground controls out to L + Day 9. There were no significant changes in total cellular protein or total DNA content in the flight samples compared to time-matched ground controls; but during the 10 days of the experiment, there was substantial cell proliferation as indicated by a 55%-60% increase in DNA content ($P<.001$) in both flight and ground cultures. Total protein content increased to a similar extent in both groups, resulting in a constant protein/DNA ratio. The muscle specific protein, myosin heavy chain, also significantly increased in content during this time period (42%, $P<.05$), but only in the ground controls. In contrast, the content of the extracellular protein fibronectin significantly decreased (29-33%, $P<.05$) during this time period in both flight and ground controls. **Space flight therefore specifically inhibited the accumulation**

[1]This difference may be due to a protective collagen coating placed around the STS77, but not the STS66, muscle cells pre-launch. In STS66, the muscle cultures were transferred to the STL perfusion cartridges on Day 11 postplating and launched into space on Day 14. In STS77, the cell cultures were four days younger when loaded into the STL cartridges (Day 7 postplating) and launched on Day 11 instead of Day 14. The younger age of the cell cultures used in STS77 was due to the fact that our first set of muscle cultures initiated 18 days before the STS77 launch (after a 3 day launch slip) did not look as healthy as our backup cultures initiated 11 days before launch. We therefore decided to go with the healthier looking, but younger muscle cultures. In order to better protect these more fragile cells during transportation to KSC and launch, we decided to "collagen-embed" the muscle cells, a common practice in our lab. The resultant muscle "organoids" used in STS77 therefore contained well-differentiated muscle fibers surrounded by a thick (>50μM) protective layer of connective tissue composed mainly of type I collagen. The muscle organoids used in STS66 contained well-differentiated muscle fibers surrounded by a thin layer of extracellular matrix (<10μM) synthesized endogenously by the fibroblasts in the cultures.

of myosin heavy chain in growing muscle fibers.

Total protein synthesis rates of rapidly turning over regulatory proteins measured at Day 9 of flight was significantly decreased in the flight samples compared to ground controls (74%, P<.01). In contrast, reloading the muscle cells on return to 1xG significantly increased total protein synthesis rates of these proteins over ground controls (77%, P<.001). **Synthesis rates of regulatory proteins are therefore down regulated in the absence of gravity and up-regulated on return to 1xG. Synthesis rates of myosin heavy chain, fibronectin, and collagen were also significantly elevated in the reloaded flight samples compared to ground controls.**

Overall these STS77 results compare well with those obtained in the STS66 experiment and to changes seen in skeletal muscle of rodents flown aboard the Space Shuttle. In both tissue culture and animal systems, neither general muscle metabolism rates nor total protein degradation rates are significantly altered by space flight, while muscle fiber size and total protein synthesis rates are reduced in flight relative to ground controls. In addition, increased cell proliferation has been noted in both animal muscle and the tissue cultured muscle organoids in microgravity. A preferential decrease in myofibrillar content is also noted in both instances. Finally, reloading of the muscle cells after flight activates the protein synthetic process in both *in vitro* and *in vivo* muscle. **While caution must be used in directly comparing the response to microgravity of fully differentiated muscle in animals to partially differentiated muscle in tissue culture, the results indicate that tissue cultured skeletal muscle organoids provide an excellent *in vitro* model for developing countermeasures to prevent skeletal muscle loss in astronauts during long-term space travel.**

STS 77 Experimental Results

1. Cell Metabolism in the STL/A Module

Summary -**Metabolism rates, based on medium glucose/lactate levels, were similar in flight and ground samples, both during the launch period and while in space.**

Daily measurements of perfusion medium glucose/lactate levels were made starting L-3 Days and continued to L+9 Days on STL cartridges, each containing 6 muscle "organoids." The cells were metabolically active in the glucose-enriched Dulbecco's modified Eagle's medium (DME, 4.5 g/L glucose), buffered with HEPES and containing 2.5% (v/v) horse serum. They utilized approximately 24% of the total glucose available in the 155 ml of media during 13 days in the cartridges, with an equivalent increase in lactate concentration in the media. The rates of glucose utilization (0.08 g/L/day) and lactate accumulation (0.07 g/L/day) in the medium from L-2 Days to L+10 Days were similar in both groups (**Figures 1 and 2**). Rates of metabolism are slightly higher than those in STS66, possibly due to the younger age of the cultures. During the launch period (L-12 Hour to L+12 hours) the rates of glucose utilization and lactate production were similar in both groups (**Figures 1 and 2 insets**).

The most important conclusion from these results is that space travel had little effect on the overall metabolic rate of the skeletal muscle tissue and indicates the ability to maintain differentiated skeletal muscle fibers in space flight studies for up to thirteen days. The results also show that the skeletal muscle cells were not "damaged or stressed" during launch when grown with a protective connective tissue coating (see Footnote 1).

2. Protein Degradation Rates

Summary - Total protein degradation rates, based on the release of radioactivity from cellular proteins pre-labelled with ^{14}C-phenylalanine, were similar in flight and ground samples both during launch and in space.

Media fractions collected during the experiment were processed for trichloroacetic acid (TCA) soluble radioactivity, and the rate of protein breakdown calculated from the percent radioactivity remaining in cellular proteins at varying times in the STL/A module. A semilog plot of the data indicates the rate at which proteins were broken down. Overall, the rate of protein degradation was very similar in ground and flight samples (**Figure 3;** protein half-life ($t\text{-}_{1/2}$) = 241-248 hours). There was no significant difference in protein breakdown rates during launch (**Figure 3 inset**) These results again show the ability to maintain tissue culture muscle cells under space flight conditions.

3. Protein Synthesis Rates In flight (L+9 Days) and Post Flight (R+5 to 15H)

A. Protein Synthesis Rates In Flight On L+9 days

Summary - Total protein synthesis rates in flight were significantly decreased (74%, P<.01) compared to time-matched ground controls, indicating a down regulation in the synthesis of rapidly turning over regulatory proteins.

Total protein synthesis rates were measured with ^{3}H-phenylalanine during a 6 h period on L+9 Day of flight. The cells were perfused with TCA after the 6 hour pulse and processed for TCA - insoluble radioactivity when returned to the ground. There was a significant reduction in the flight protein synthesis rates compared to time matched ground controls (**Figure 4**), in contrast to no

differences in metabolism and protein degradation rates between the two groups. This short radioactive pulse time will primarily measure the synthesis rates of rapidly turning over proteins and is not indicative of the general protein synthesis rates of structural proteins such as the contractile proteins. In contrast to these results, no significant difference was noted in protein synthesis rates in flight samples on Day 8 of STS66. The difference may be due to the different age of the cultures, different flight time when protein synthesis was assayed, or the different culture methods used in the two experiments.

B. Protein Synthesis Rates Following Recovery (R + 5 to 15H)

Summary - Total protein synthesis rate during reloading of the flight organoids was significantly elevated compared to ground controls (77%, P<.001). Synthesis rates of myosin heavy chain, fibronectin, and collagen were all significantly elevated during reloading.

We had difficulty obtaining accurate individual protein synthesis rates from the in-flight TCA fixed samples from STS66, and therefore changed the protocol for STS77 to pulse several cartridges with ^{3}H-phenylalanine (^{3}H-phe) during the last hours of the flight, and process the samples by the more traditional method of homogenization and freezing for subsequent polyacrylamide gel electrophoretic separation of a skeletal muscle specific protein (myosin heavy chain) and several extracellular matrix proteins (collagen and fibronectin). The Coomassie blue stained gel bands were quantitatively analyzed with an image analysis system (JAVA, Jandel Scientific) and the bands cut from the gels , solubilized and radioactivity determined by liquid scintillation counting. Because of STL hardware failure, the ^{3}H-phe was not injected into the flight cartridges pre-landing as expected. When this was discovered at R + 3H, it was decided to inject the ^{3}H-phe for the subsequent 10 hours

(R + 5 to 15H) and measure protein synthesis during this early period of cell "reloading" in 1 x G. The STL unit was maintained at 37°C during this period. Both flight and ground controls were thus pulse labelled for 10 hours, but because ground controls were injected with ^{3}H-phe on time by the computerized STL program, there was a 9 h time differential between processing the two groups.

Reloading of the muscle cells significantly stimulated total protein synthesis (77%) by an amount similar to the in-flight decrease (74%) seen approximately 30 hours earlier. This increase suggests a rapid up-regulation of the synthesis of rapidly turning over regulatory proteins. These regulatory proteins could be in either in the postmitotic muscle fibers or the connective tissue fibroblasts. When individual structural proteins were isolated by polyacrylamide gel electrophoresis, the muscle sarcomeric protein myosin heavy chain synthesis rate was significantly increased (43%, $P<.01$) and the extracellular matrix protein synthesis rates for collagen and fibronectin were also significantly increased (32%, $P<.01$; 69%, $P<.001$) relative to ground controls (**Figure 5**). Based on ^{3}H phe incorporated into total protein from R+5 to 15 hours (**Figure 4**) to ^{3}H-phe incorporated into myosin heavy chain during this time period (**Figure 5**), myosin heavy chain synthesis accounts for approximately 2% to 3.5% of the total protein synthesis in a 10 hour pulse period. **These results indicate up-regulation of contractile protein synthesis as well as total protein synthesis in the reloaded samples.**

4. Total Non-collagenous Protein, Myosin Heavy Chain, and DNA Accumulation

Summary -Total non-collagenous protein levels in samples fixed with TCA on Day 9 of flight were reduced by 16% in the flight samples compared to time-matched ground controls. DNA content was similar in both groups. Comparison of pre-flight (launch

controls) and post-flight samples (Recovery +15 H) show significant increases in both non-collagenous protein and DNA content (55% to 60%), indicating that cell proliferation occurred in both flight and ground-based controls. Since protein/DNA ratios were similar in all groups, the majority of the increase in total noncollagenous protein resulted from cell proliferation of fibroblasts rather than changes in muscle protein content. Myosin heavy chain content was significantly increased (42%, P<.05) in R + 15H ground controls, but not flight samples, when compared to launch controls. In contrast, total fibronectin content was decreased in both groups compared to pre-flight controls. These data indicate a specific inhibitory effect of space travel on a muscle specific contractile protein.

Total noncollagenous protein and DNA values were assayed by two different procedures. In a repeat of the procedure used in STS66, two cartridges in each group were fixed on Day 9 of flight by infusion of trichloroacetic acid (TCA) fixative and processing the fixed cells after recovery of the samples on Day 10. As shown in **Figure 6**, total noncollagenous protein and DNA values measured in this manner were reduced by 16% and 11%, respectively. Protein/DNA ratios were similar in the two groups (**Figure 7**). This small, nonsignificant decrease in total protein content in the STS77 flight samples is in contrast to the increase reported in STS66. As noted in our final STS66 report, their were large variations in the values obtained from the two STS66 flight cartridges, and we increased our sample size in STS77 because of this variation. We believe that the data obtained from STS77 is more reliable because of the larger number of samples in each group, and the fact that two different assay methods were used in STS77 (see next paragraph) and both agree with each other.

In a second group of cartridges, the cells were processed at R + 15 hours by more standard

laboratory techniques (rinsing cells in ice cold saline, freezing, and homogenizing before TCA precipitation of proteins at 4°C). This protocol was identical to that used pre-flight on launch controls so a comparison could be made of pre-flight and post-flight samples in STS77. As shown in Figure 8, there were significant increases in both total noncollagenous protein and DNA compared to the launch controls. Protein content increased 66% and 62% in ground and flight samples, respectively ($P < .002$), while DNA content increased 60% and 55%, respectively ($P < .001$). There were no significant differences between the Day 10 flight and time-match ground samples since the protein/DNA ratio was quite similar in both groups (**Figure 9**). These data indicate that there was significant cell proliferation in the STL Unit during the ten days of the experiment, and that the majority of protein increase in the muscle organoids resulted from this cell proliferation rather than changes in the muscle fibers. The protein/DNA ratios increased 8% and 5%, compared to launch controls, in Day 10 ground and flight samples, respectively, (**Figure 9**) and these increases were significant ($P < .05$). This indicates that there was a small but significant degree of hypertrophy in the postmitotic muscle cells, and that the hypertrophy was slightly less in flight samples compared to ground controls.

Myosin heavy chain (MHC) and fibronectin protein content were determined by quantitative polyacrylamide gel electrophoresis on pre-flight and post-flight samples. MHC content increased significantly (42%, $P < .05$) in R + 15H ground control samples compared to pre-launch controls, indicating that the muscle fibers were accumulating muscle specific proteins during the experiment (**Figure 10A**). In contrast, R + 15H flight samples contained 21% less MHC than the time matched ground controls and this was not significantly different than launch control samples. In comparison, the accumulation of the extracellular matrix protein fibronectin, was significantly decreased (29%

to 33%, P<.05) in both ground and flight samples when compared to launch controls (**Figure 10B**). **These data indicate a specific inhibitory effect of space flight on myosin heavy chain accumulation in growing muscle cells.**

5. Myofiber Diameter Measurements

Because of the pre-flight embedding of the muscle cells in a collagen matrix (see Footnote 1), standard staining techniques for performing quantitative morphometric analyses of the muscle fiber diameters has not been successful. We will perform a more laborious method of embedding the cells in Epon and thin sectioning the samples in an attempt to obtain this data from the STS77 samples. In STS66, a small but significant atrophy of the flight muscle fibers was noted (10%, P<.01).

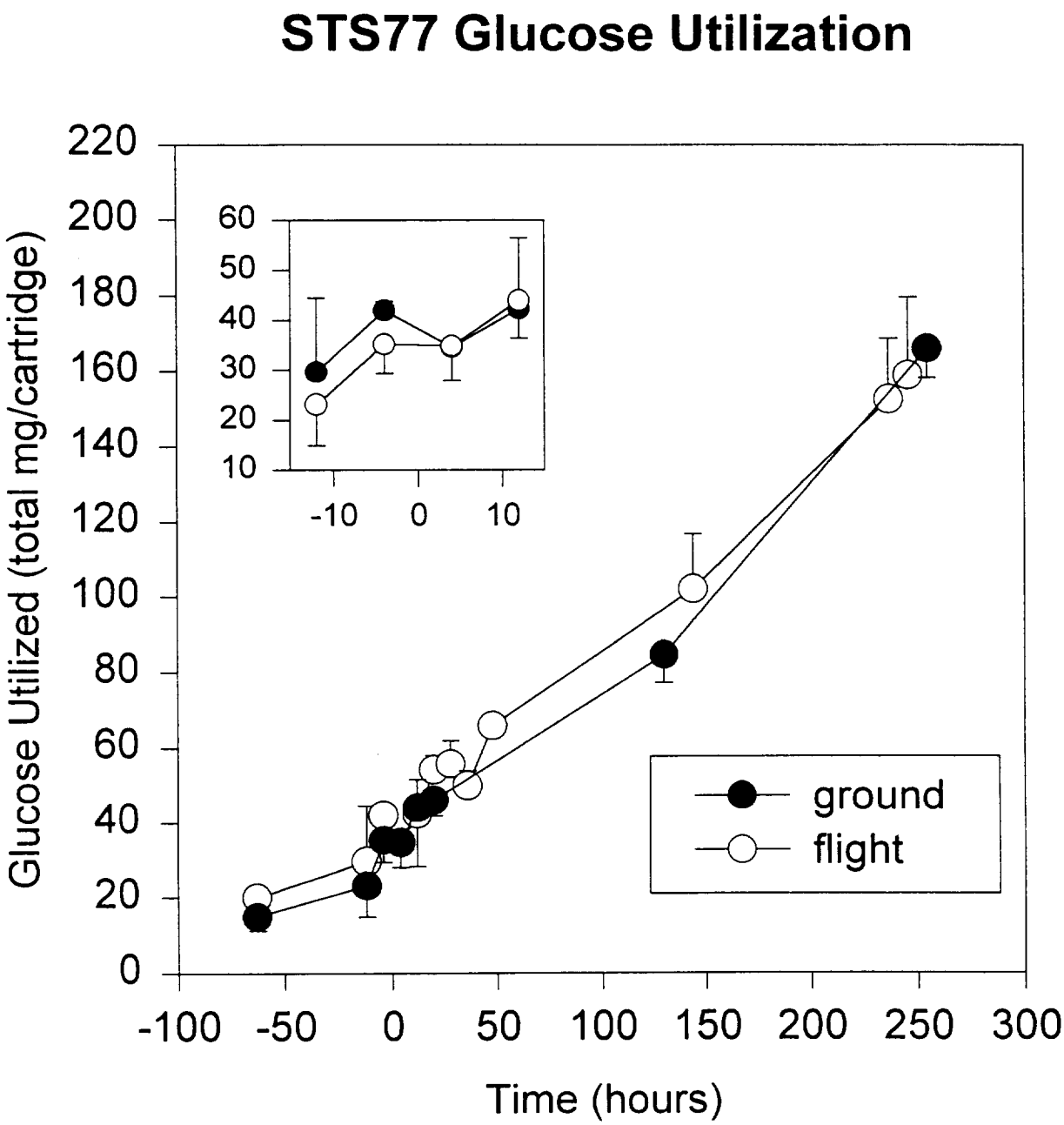
6. Autocrine Growth Factor Secretion

Summary - Accumulation of the autocrine muscle growth factor prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in the medium was increased by space flight, but not significantly. Measurement of a second autocrine muscle growth factor, insulin-like growth factor-1, is underway.

Fraction collected samples were returned to our lab from KSC at 4°C and aliquots removed for measurement of $PGF_{2\alpha}$ by ELISA. Flight definition studies indicated that $PGF_{2\alpha}$ is stable in the medium under conditions of the STL unit (37°C for 8 to 10 days). $PGF_{2\alpha}$ is not present in the initial culture medium containing 2.5% serum, and secretion increases rapidly when the muscle cells were placed in the STL unit (**Figure 11**). $PGF_{2\alpha}$ secretion plateaued early post launch in both flight and

ground-based controls and no significant launch-induced increase in $PGF_{2\alpha}$ secretion was noted, although the values tended to be higher in the flight samples compared to the time-matched ground controls. Because of STL fraction collector problems, only eight of twelve samples were collected in the flight unit postlaunch, and only 2 of these were after L+ 36 hours. There was no significant difference between flight and ground controls in these samples. These results are similar to those obtained in STS66, with the exception that $PGF_{2\alpha}$ levels increased more quickly in the STS77 experiment than in STS66. This may be related to the greater proliferation of the fibroblasts in STS77 than in STS66, and their ability to synthesize and secrete prostaglandins.

FIG. 1



GLUgr2.SPW

FIG. 2

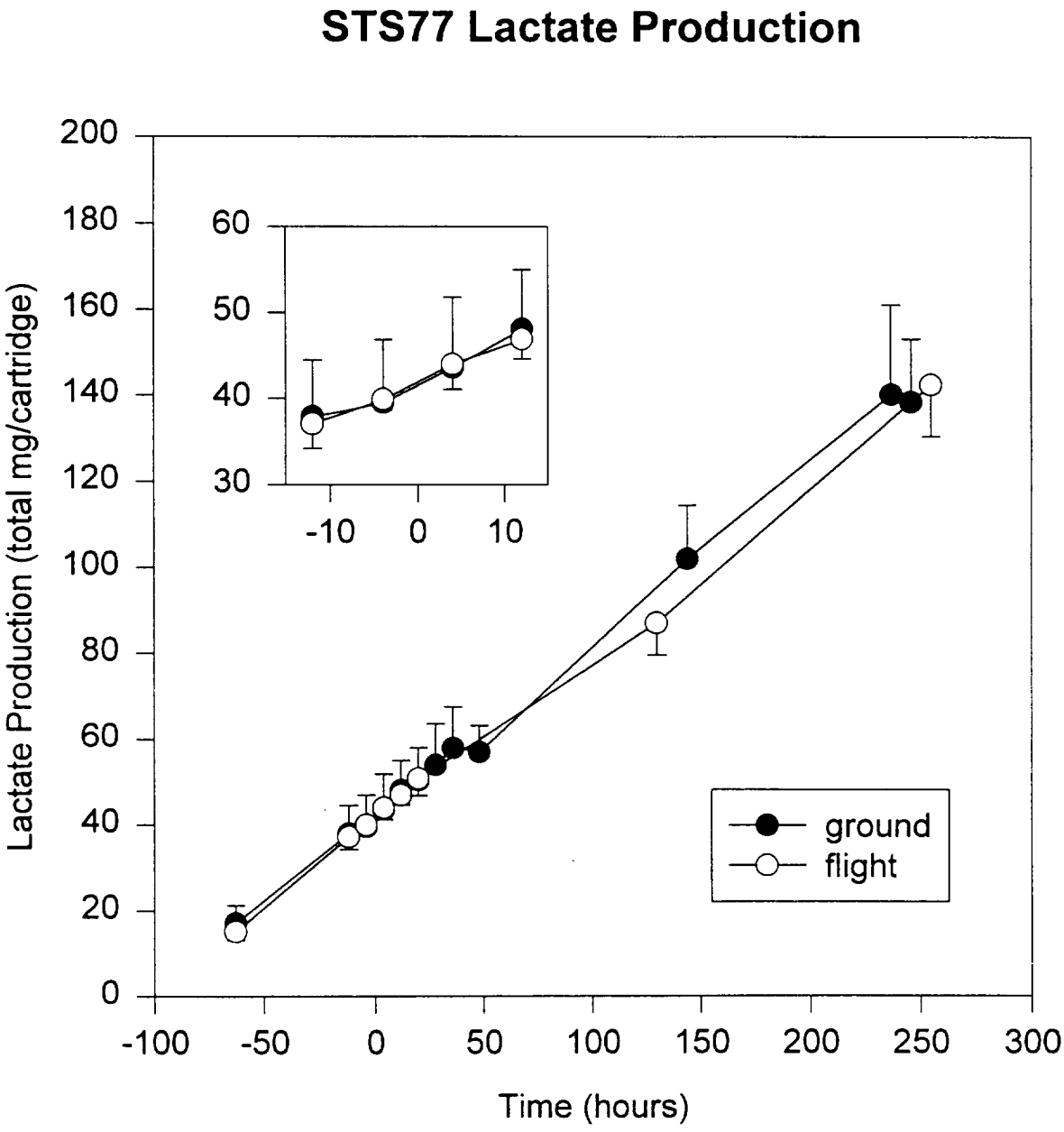


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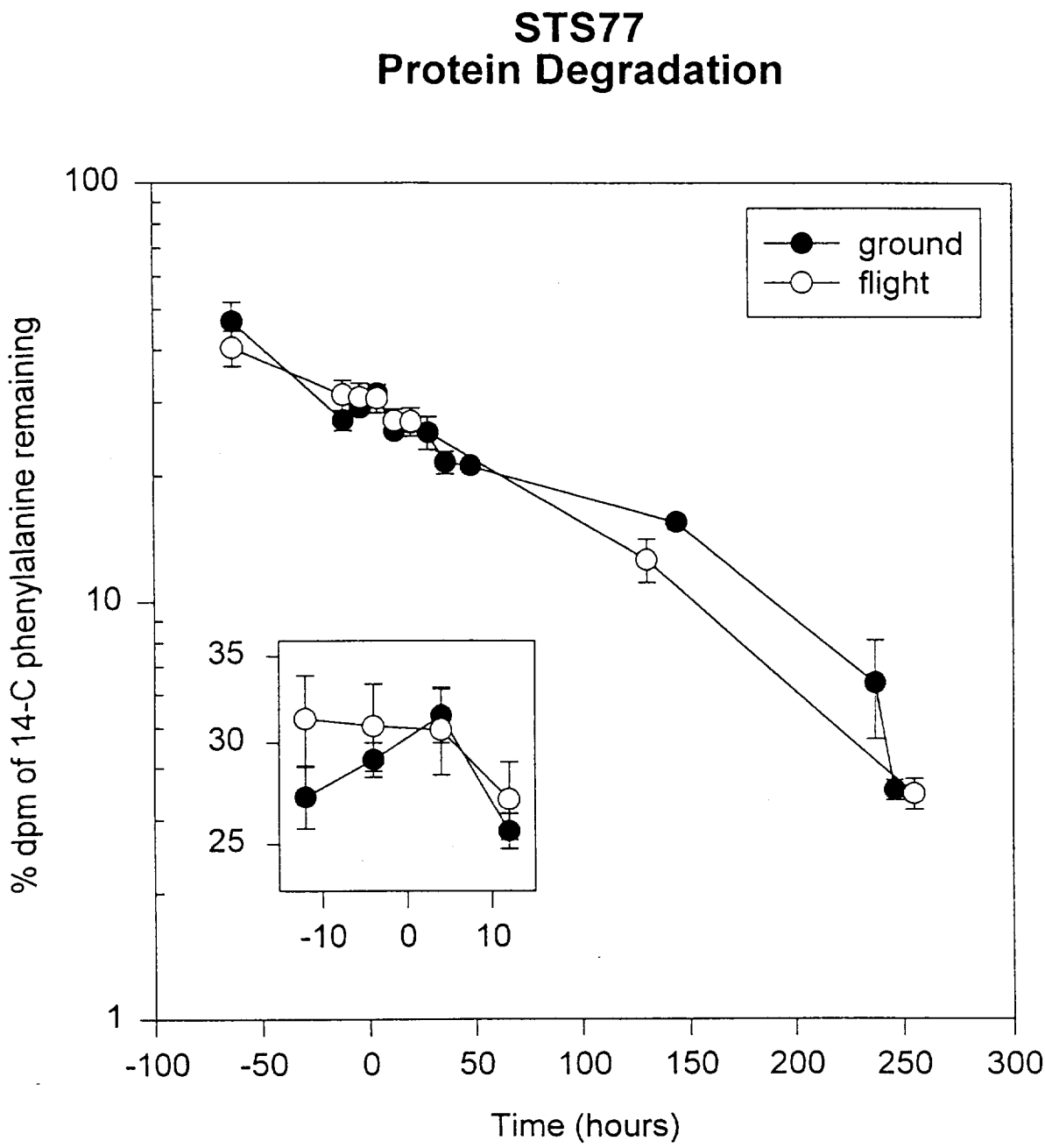
FIG. 3



deggrph3.spw

Figure 4

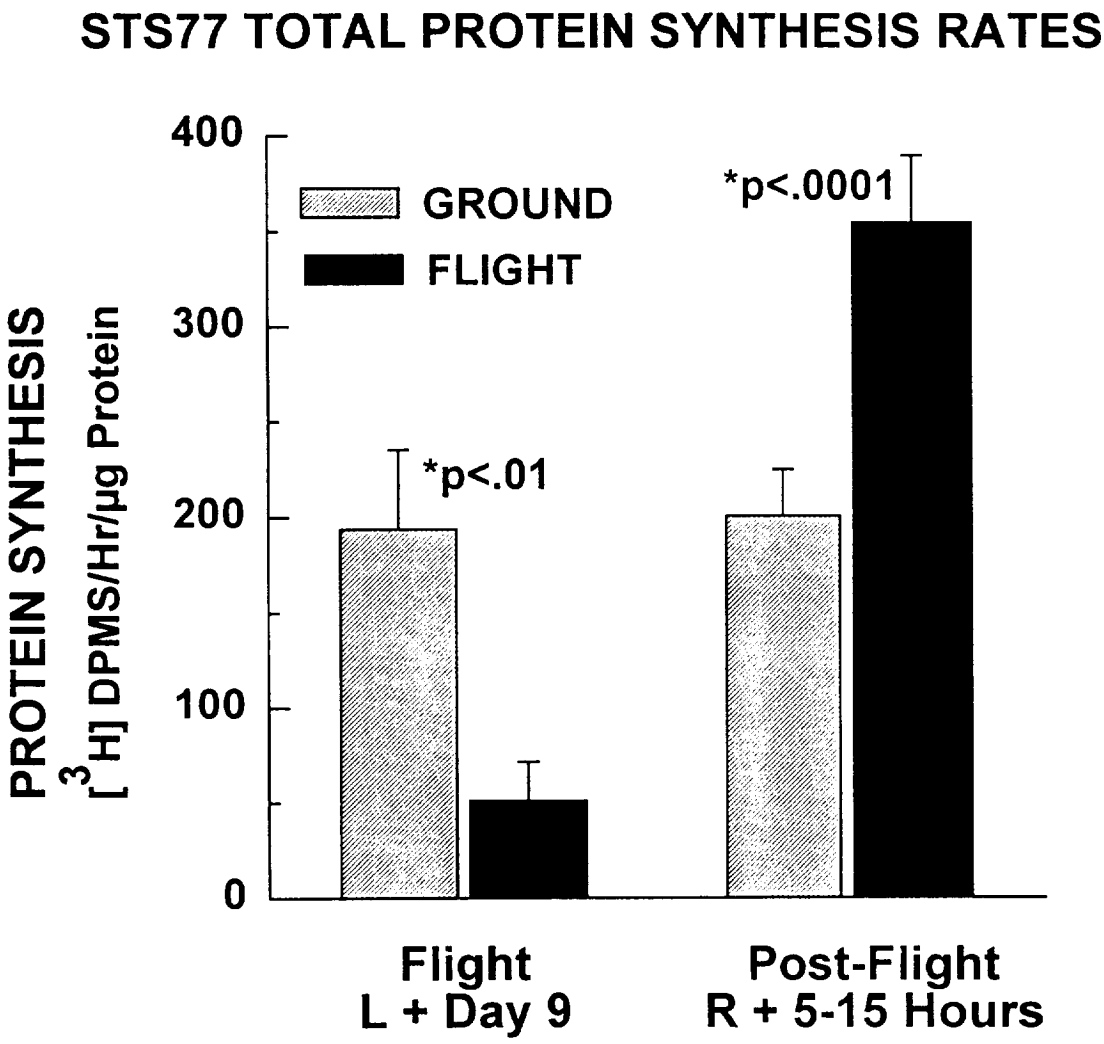

STS77 TOTAL PROTEIN SYNTHESIS RATES
PROTEIN SYNTHESIS
[3H] DPMS/Hr/µg Protein
400
300
200
100
0
GROUND
FLIGHT
*p<.01
*p<.0001
Flight
L + Day 9
Post-Flight
R + 5-15 Hours

Figure 5

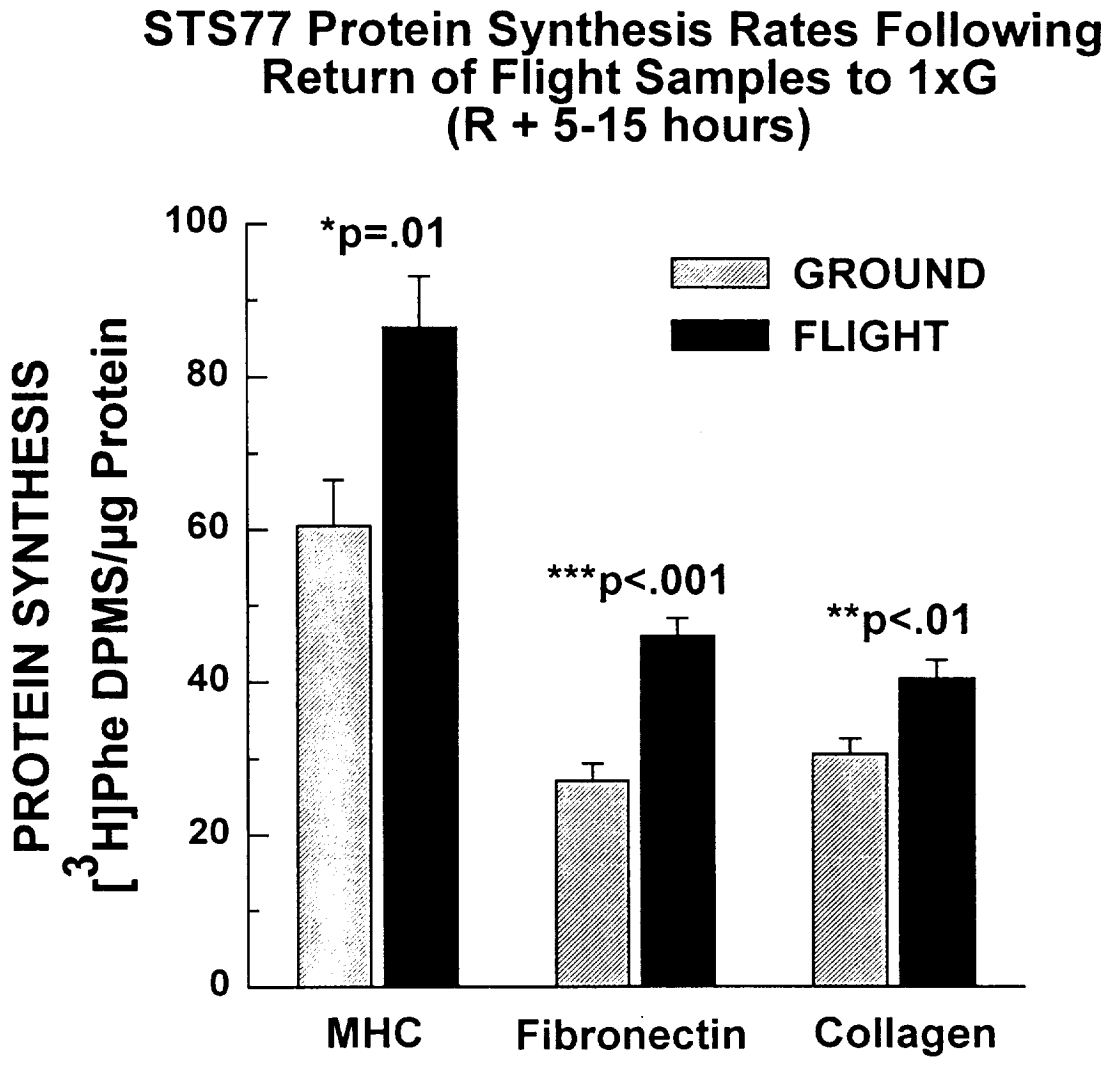
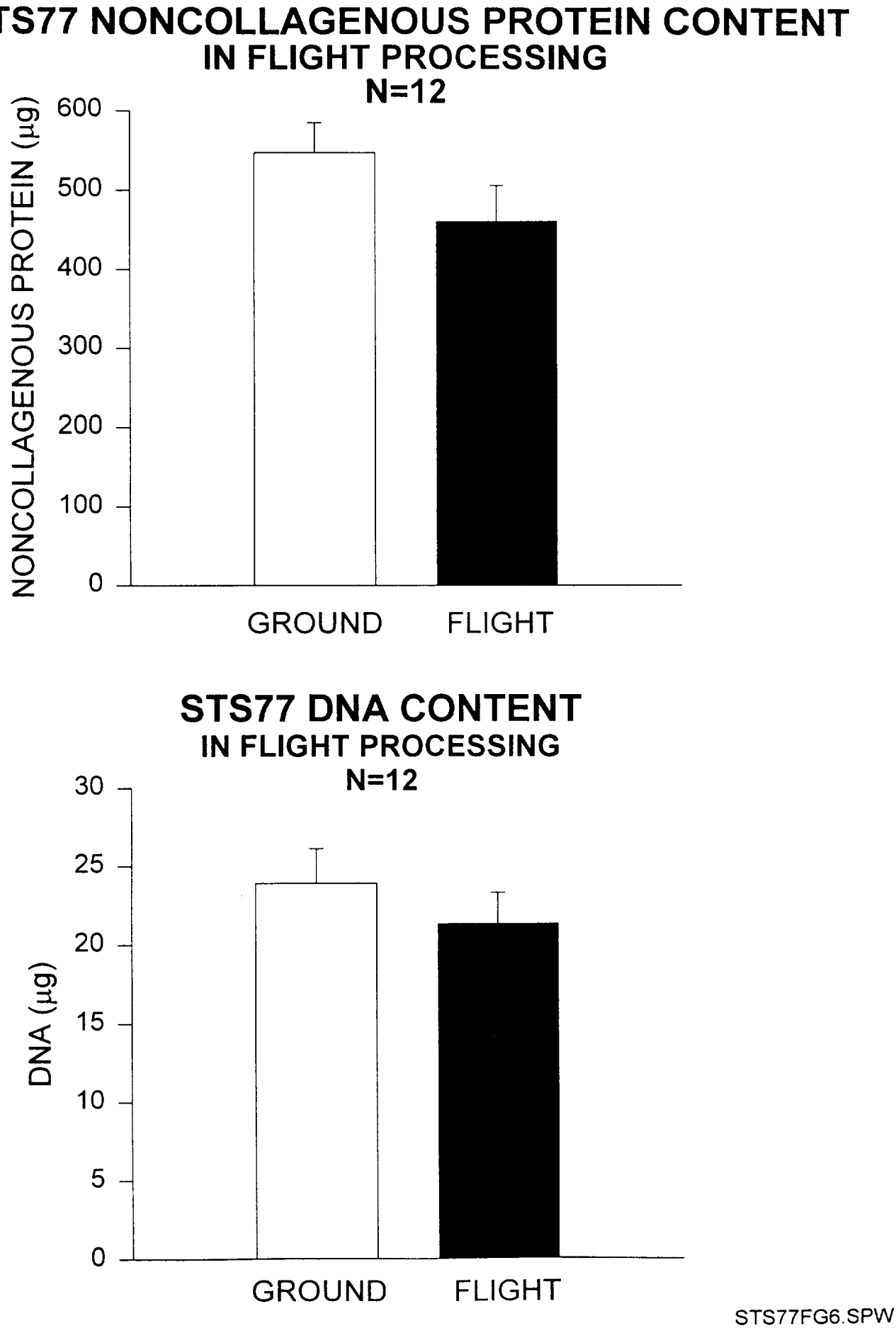
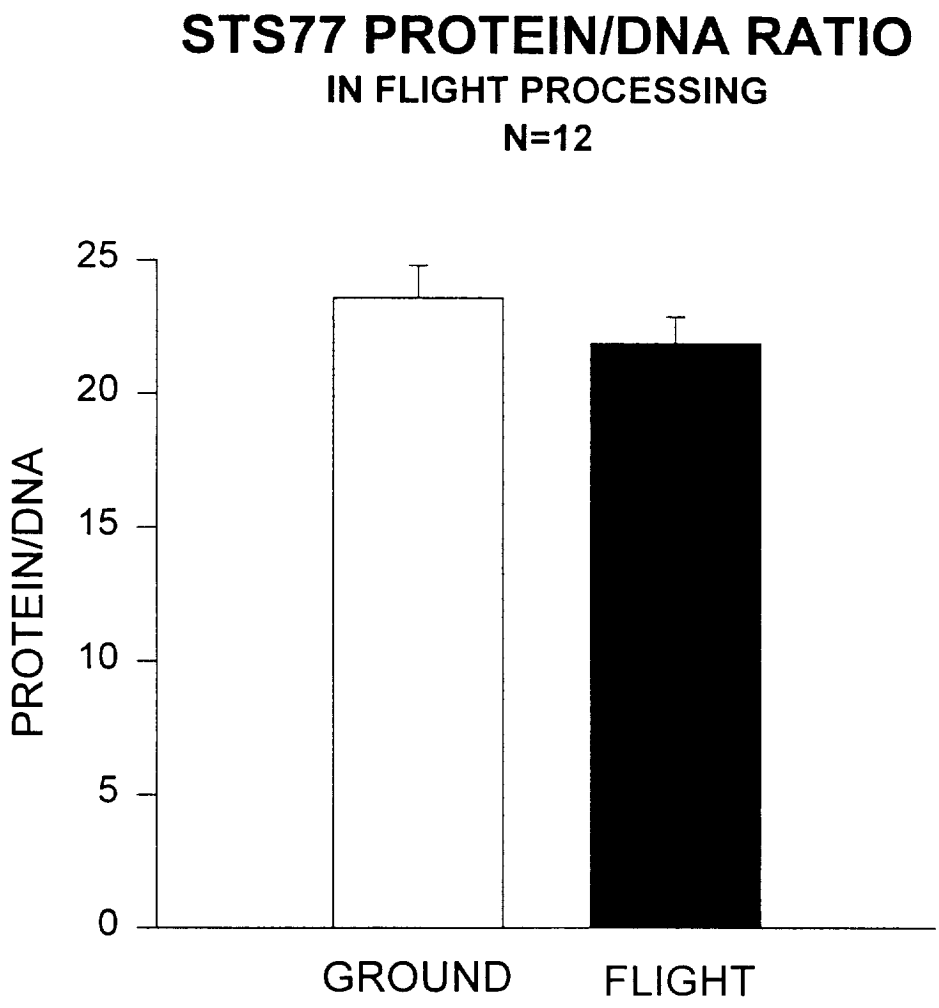

STS77 Protein Synthesis Rates Following
Return of Flight Samples to 1xG
(R + 5-15 hours)
PROTEIN SYNTHESIS
[3H]Phe DPMS/µg Protein
100
80
60
40
20
0
*p=.01
***p<.001
**p<.01
GROUND
FLIGHT
MHC
Fibronectin
Collagen

Figure 6



STS77FG6.SPW

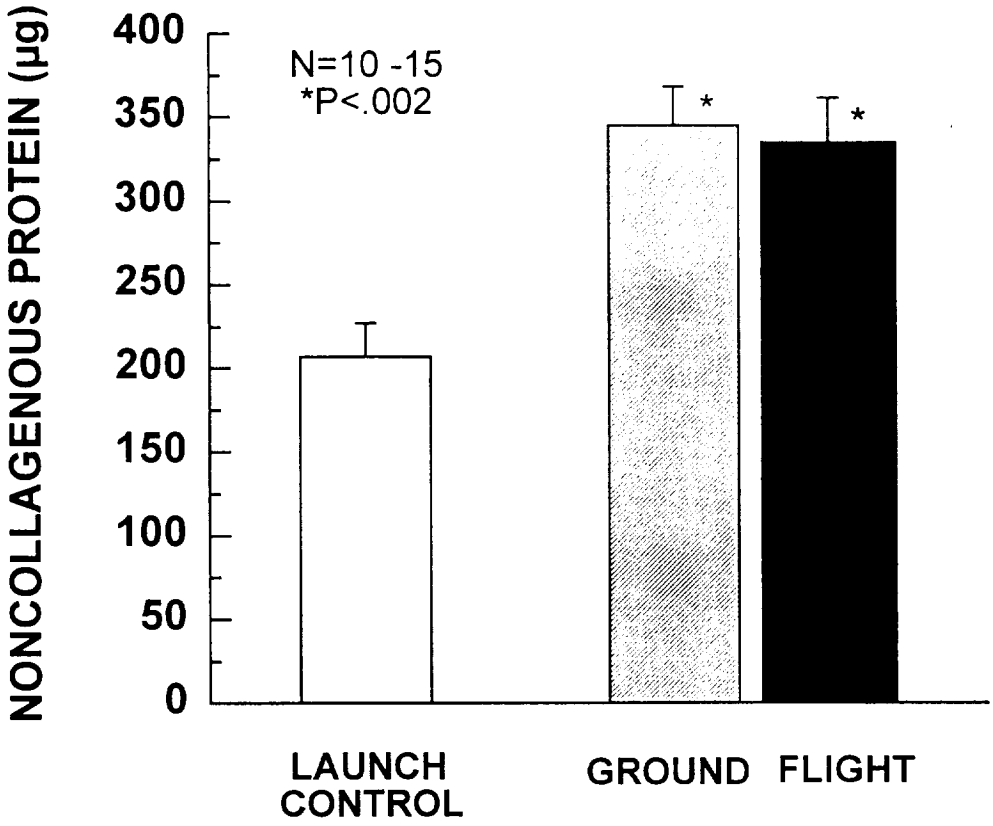
Figure 7



STS77FG7.SPW

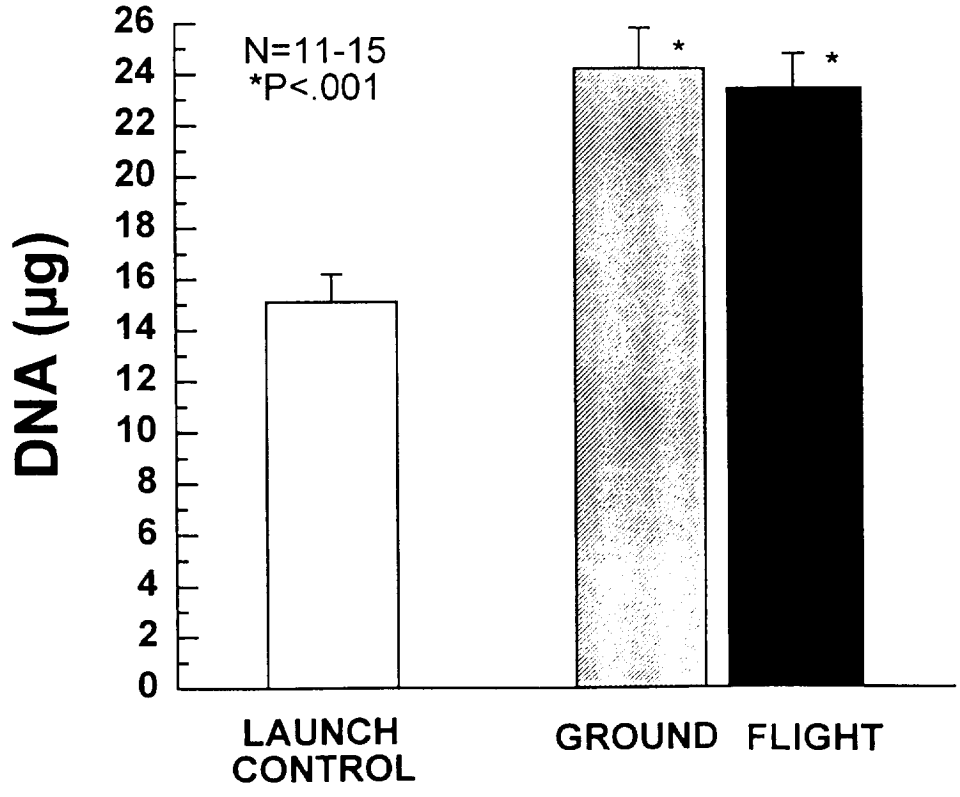
STS77 NONCOLLAGENOUS PROTEIN CONTENT

POST FLIGHT PROCESSING



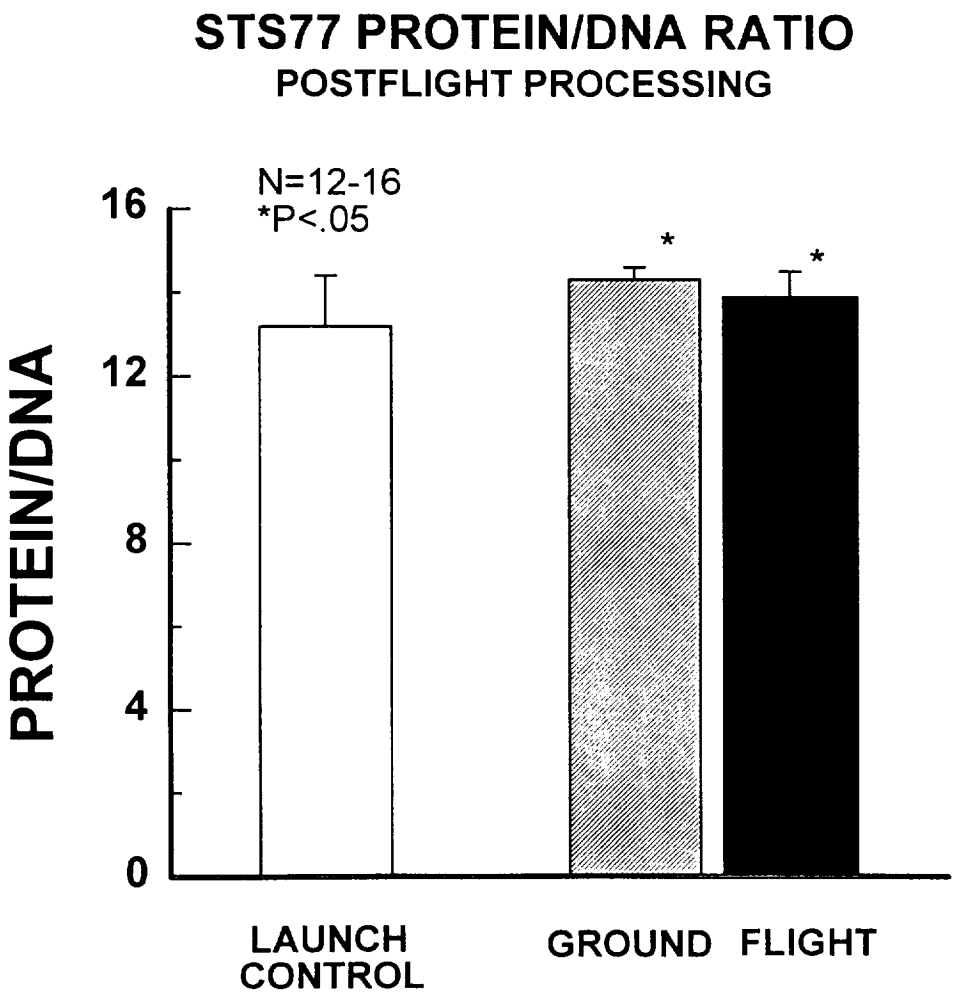
STS77 DNA CONTENT

POSTFLIGHT PROCESSING



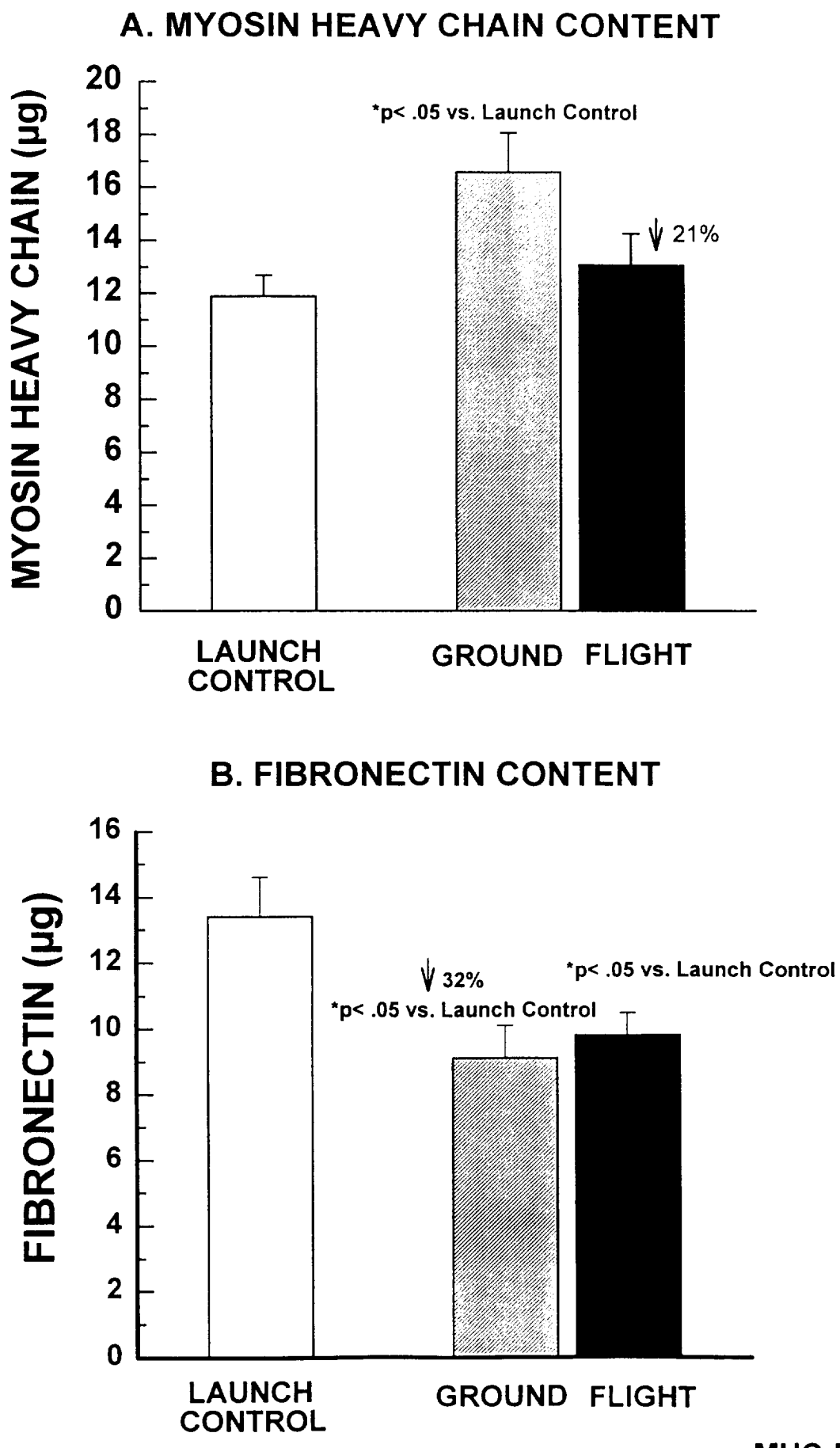
PRTN.SPW

Figure 9



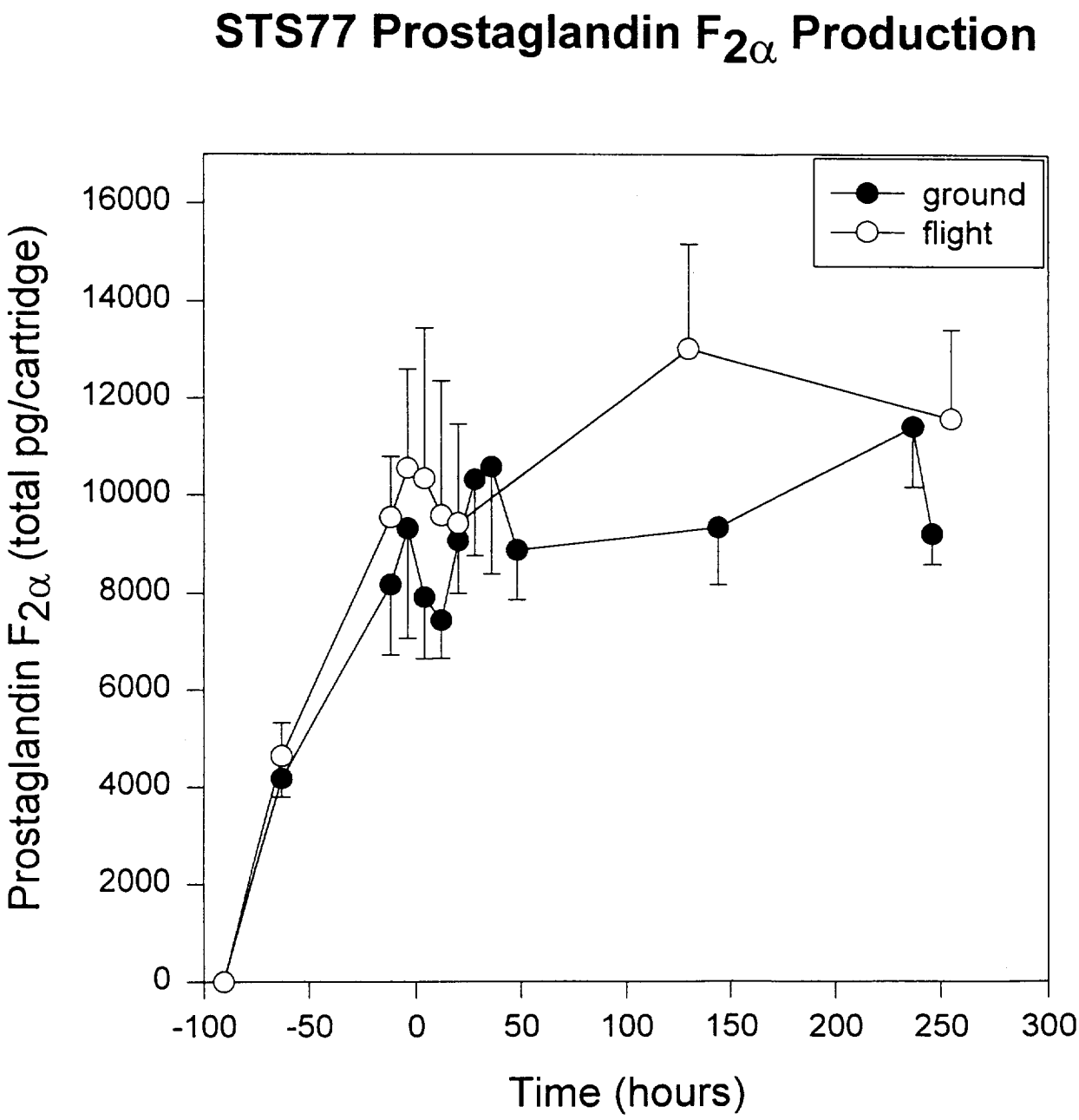
PRODNA.SPW

Figure 10



MHC-FBA.SPW

Figure 11



f2asts77.spw
7/16/96