5-2012

Effects of Microgravity on Mucin Production in the Urinary Bladder in Mice.

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EAST TENNESSEE STATE UNIVERSITY

MIDWAY HONORS SCHOLARS PROGRAM

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May 2012

A Senior Honors Thesis Submitted as a Partial Fulfillment of the
Midway Honors Scholar Program,
East Tennessee State University, Johnson City, TN

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Abstract:

The effects of the microgravity of spaceflight are largely unexplored with regard to biological tissues. One particular area of interest is the possible effects microgravity could have on the production of mucins. To determine the possible effects of microgravity on mucin production in the urinary bladder, we examined the transitional epithelium of the urinary bladder from female mice that were flown on the space shuttle Endeavour for 12 days in August, 2007. The flight tissue was compared to tissues from two control groups of animals, ground control and baseline. This study utilized three sets of female mice, with each set consisting of 12 animals. The three sets were designated as Flight, Ground Control, and Baseline. The flight animals were flown in the Commercial Biomedical Testing Module-2 (CBMT-2) which was housed in the shuttle's mid-deck locker area. Ground control animals were also housed in CBTM-2 units which were kept in environmentally controlled rooms at the Space Life Sciences Lab at Kennedy Space Center. Baseline animals were also housed at the Space Life Sciences Lab but were housed in standard rodent cages with ambient temperature and humidity, with a 12/12 light dark cycle. Bladder tissue was paraffin embedded, sectioned, mounted, and histologically stained using an Alcian Blue Periodic Acid Schiff staining procedure. The bladder tissue from the three treatment groups is being qualitatively analyzed for mucin thickness and types of mucins produced. To date the study indicates that the mucin layer of the Flight tissue is thinner than that of the Baseline or Ground Control tissue, but only significantly thinner than the Baseline tissue.

Introduction:

A mere century ago space flight was a figment of the human imagination. Over the past century there has been great advancement in the field of space flight. As space exploration evolved, the ability for mammals, including humans, to travel to space has been made possible. By 1950, the United States had launched two primates into space aboard V2 rockets (Kohn, 1991). Neither animal survived its mission, but useful information about the hazards of mammalian spaceflight was obtained. Thus, spaceflight medical research was born. Having mammals travel to space and back to earth intrigued the research community to explore what effect spaceflight has on living systems. During the 1950s, medical scientists became more interested, and identified many potential problems for astronauts that could be crucial during a space mission. Anorexia, nausea, inability to swallow food, and disorientation, were among thirty predicted effects of "weightlessness" (Kohn, 1991). After the Project Mercury mission, the six astronauts returned in satisfactory health, but did reveal two principal findings, weight loss due to dehydration, and cardiovascular impairment (Kohn, 1991). Both dehydration and the cardiovascular impairments have since been linked to fluid shifts that occur in the body. The next NASA project was called Project Gemini. This mission began with increased objectives and capabilities. Gemini tested the abilities of astronauts to survive for longer periods of time in space, and even outside of the spacecraft on lunar landing missions (Kohn, 1991). After the ten manned space missions of Project Gemini, the principal concern of the medical investigators was changes in cardiovascular function (Kohn, 1991). Cardiovascular deconditioning became the focus of research and investigation during the Project Apollo. Following the completion of the Apollo missions, cardiac arrhythmia was added as a significant biomedical finding (Kohn, 1991). Researchers continued to study the possible causes of these cardiovascular problems; and with

the development of Skylab, scientists were given the first opportunity to study long term effects of spaceflight (Kohn, 1991). Skylab gave astronauts the ability to stay in space for several months at a time. Due to the findings of the previous Gemini and Apollo missions, cardiovascular deconditioning was given particular attention in Skylab research. Orthostatic tolerance, electrical activity, and changes in heart size were closely observed (Kohn, 1991). Another important observation that was made during the Skylab missions was a significant increase in the excretion of urinary calcium during flight for all crewmembers (Kohn, 1991; Whitson, et. al., 2001). This calcium loss continued throughout flight, without evidence of decreasing during later stages. This calcium loss was attributed to marked loss in leg volume, and to body fluid deficit. Skylab was crucial in providing scientists with the ability to distinguish self-limiting physiological changes from those that seem to persist throughout the longer missions (Kohn, 1991). In Earth-based and space-based experiments, prolonged exposure to microgravity has been shown to negatively affect the functional capacity of tissues and cells throughout the body. Some of the tissue systems affected are the skeletal system (Droppert, 1990, Milstead, et al. 2004), Quail oviduct length (Skrobanek, et al. 2008), Seminiferous Tubules (Kamiya, et al. 2003, Motabagani, 2007, Forsman, 2012), the immune system (Armstrong, et al. 1993, Chapes, et al. 1993, Chapes, et al. 1999), skeletal muscle arterioles and regional blood flow (Arbeille, et al. 1996, Delp, 1999), as well as overall body fluid shifts (Tipton, et al. 1987). As the space program expands and requires astronauts to remain in space for extended periods of time, it is important to understand the physiological effects of microgravity on all tissues of the body.

Cosmic Radiation:

One major obstacle to human space exploration is the possible limitations imposed by the adverse effects of long-term exposure to cosmic radiation. Exposure to high-energy, ionizing cosmic ray (HZE) nuclei is dangerous to humans and test animals because of the ionizing effect it exerts on atoms and molecules (Setlow, 2003). The biological effects of HZE nuclei on cancer induction, the central nervous system, the immune system, and the eyes are not well known (Setlow, 2003). The interaction of radiation effects and exposure to microgravity needs to be investigated to better understand the role of each in physiological changes experienced while in space. The physiological changes that have been documented from the beginning of spaceflight could be attributed to cosmic radiation. It is essential to better understand cosmic radiation and ways to shield astronauts to better protect them while in spaceflight missions.

Fluid Shifts:

It has been repeatedly documented that most astronauts and cosmonauts lose body mass during space flight with the majority of this loss being body water (Tipton, et. al., 1987). Part of the decrease in total body water is related to a decrease in water intake, a reduction in the amount of metabolic water being formed, and to sweating. The first change in the cardiovascular system in weightlessness occurs within the first hours of flight. Fluid shifts to the upper body to yield facial edema and venous distention. Most authorities would agree that the transition from earth's gravity to a microgravity environment is the primary stimulus for the fluid shifts and their cascading effects on bodily appearances and functions (Tipton, et. al., 1987). To understand why fluids shift to the thorax and neck of the body during spaceflight, a quick review of the cardiovascular system is necessary. The cardiovascular system of humans and animals was

designed to function here on earth. The cardiovascular system was designed to pump fluids from the extremities back to the heart. On earth, the system has to push those bodily fluids against gravity. However, in space the effects of gravity are negligible; thus gravity is not fighting against the cardiovascular system. This gives fluids the opportunity to accumulate in the thorax and upper region of the body. The loss of body water can be coupled with girth decreases in the thigh and calf regions (Tipton, et. al., 1987). Studying fluid shifts has proven to be difficult, due to the inability to standardize an animal model. Also, fluid shifts can be influenced by anxiety, stress, and other medical issues that cannot be eliminated (Tipton, et. al., 1987).

As previously stated, astronauts experience a loss of body water. One of the principle causes of water loss is a decrease in water intake. Astronauts, due to fluid shifts, do not experience the feeling of being thirsty as they would on earth. As a result of not becoming thirsty and not drinking, the astronauts become dehydrated. When an individual becomes dehydrated their body has to retain as much fluid as it can. This increases the concentration of urine, and also decreases urine output (Whitson, et. al., 2001; Monga, et. al., 2006). These changes can have potential effects on the bladder, and that is one of the focuses of this study.

Urinary Bladder Anatomy:

The bladder of mice is similar to the human bladder in several structures. Like the human urinary bladder, the bladder of mice has rugae which allow the bladder to expand to hold more fluids. When the bladder is not distended, the rugae created folds in the interior of the bladder. It is on this interior surface where mucins are found in the bladder. Mucins serve a protective function, creating barriers against contact with potentially hazardous materials. Within the epithelium of the urinary bladder, the secreted mucins help protect the lining of the

bladder from potentially hazardous waste products and the changing pH of urine. Also, like the urinary bladder of the human, the urinary bladder of the mouse contains a trigone region in the lower portion of the bladder near the urethral opening (Viana, et. al, 2007). The trigone is a structurally different region of the bladder, and had to be accounted for in this experiment because the mucin layer located in the trigone may be of a different thickness than the rest of the bladder.

Mucins:

Mucins are a family of large, heavily glycosylated proteins (Bowen, 1998). Since mucins are very densely covered with sugars, this gives them a considerable water-holding capacity and makes them resistant to proteolysis. Some mucins are membrane bound due to the presence of a hydrophobic membrane-spanning domain that favors retention in the plasma membrane (Bowen, 1998). Mucins are hydrophilic because they are secreted onto mucosal surfaces. Mucins have a protective function, creating a barrier against contact with potentially hazardous materials. Mucins can be found throughout the body including the gastrointestinal tract, genitourinary tract, reproductive tract, and in salivary glands. In the aforementioned regions of the body, mucins serve several vital functions including protecting the body from pathogens and noxious substances, and acting as a lubricant to minimize shear stress on epithelium (Bowen, 1998). Mucins are produced by either a goblet cell or peg cell.

Materials and Methods:

Twelve C56BL/6 female mice (Charles River, Wilmington, MA) were flown on NASA space shuttle mission STS-118 from August 8th – August 12th, 2007. These animals were housed in the Commercial Biomedical Testing Module-2 (CBTM-2), which was part of the payload of the shuttle Endeavour's mid-deck flight locker (Figure 1). This flight duration exposed these animals to approximately 12 days of microgravity. These animals were designated as Flight (FL) animals. A second set of 12 mice were also housed in CBTM-2 units which were kept in the Space Life Sciences Lab at Kennedy Space Center. These units were subjected to the same environmental fluctuations as the flight units, with the exception of the microgravity and radiation of spaceflight and the stresses of launch and landing. In order to mimic the flight environmental conditions, these units and animals were run at a 48 hour delay in relation to the FL animals. These animals were designated as Ground Control (GC) animals. A third set of 12 mice were housed in standard rodent cages, also housed in the Space Life Sciences Lab at Kennedy Space Center on a 12:12 light:dark cycle. These animals were designated as baseline (BL) animals. The animals from all three groups were approximately 9 weeks old at the beginning of the mission. These animals were part of a tissue sharing endeavor between several researchers, each studying different tissues. The principal study was funded by the Amgen Corporation (Thousand Oaks, CA), and as such, they had some tissue of proprietary value. The dissections were carried out in different stages by different members of the research teams. The dissection procedure, in brief was conducted as follows; Amgen scientists received the live animals from the shuttle unloading team and weighed each animal, drew blood samples, and then euthanized the animals. They then removed the tissues of interest in their portion of the study. Individual sections/body organs were then brought to the other researchers so that they could

work with their particular tissue of interest. This procedure had to be conducted as quickly as possible because these animals are now back in the earth environment and the possible effects of microgravity could already be in the process of reversal. Because of this frenzied dissection, occasionally the primary dissection team would miss or accidentally destroy some tissues of secondary importance to them. In our case, some urinary bladders were lost so our portion of the study does not utilize 12 FL, 12 GC, and 12 BL mice, but rather 9 FL, 9 GC, and 10 BL. The urinary bladder tissue was removed from the animals, fixed in 4% paraformaldehyde , and paraffin embedded by Dr. Allan Forsman. Initial sectioning of the bladder tissue revealed that the paraffin did not permeate to the interior of the urinary bladder. To address this problem, the bladders were melted out of the embedding paraffin and cut in half and placed back into liquid paraffin and subjected to re-embedding. Following the re-embedding, tissue sections were cut at 4μm on a Microm HM325 microtome and subsequently mounted on glass microscope slides. The prepared slides were stained using an Alcian Blue Periodic Acid Schiff staining technique. This technique is routinely used for staining mucins because this method differentially stains glycoproteins. This method will stain the glycoproteins a deep purple color. This staining method is also useful in differentiating between acidic, basic, and neutral mucins. Acidic mucins will stain blue, basic mucins will stain red, and neutral mucins stain magenta. The tissues were visually analyzed by microscopy, using a Zeiss Axioskop 40 microscope. The use of the microimaging software, AxioVision, enabled us to photograph the tissue and make measurements of the thickness of the mucin layer. The FL, BL, and GC tissues were qualitatively analyzed, comparing mucin production, staining results, and quantitatively analyzed by measuring mucin thickness.

Six slides per bladder were prepared and three of these selected for measurements. Five random measurements were then taken from each of these 3 slides to yield 15 measurements per tissue. The average of these 15 measurements was then calculated to obtain an average mucin thickness per urinary bladder. To eliminate bias in taking measurements, we employed a randomization grid. Also, to obtain random coordinates, we employed a website that would generate 15 sets of random x, y coordinates (randomizer.org). The coordinate grid was then placed over the photograph, the random coordinate was located on the grid, and a measurement was then taken at that point. If the random coordinate fell in an area with no mucin layer, a circle was drawn to find the nearest location with a mucin layer. After all the measurements were taken, the measurements for each tissue were averaged. A one way ANOVA was used to determine if any significant difference in the thickness of the mucin layer existed between treatment groups.

Results:

The Alcian Blue Periodic Acid Schiff staining technique employed in this experiment was used because it differentially stains mucins based on their pH and would allow for better visualization of the mucins thus enabling measurement of the mucin layer. All mucins visualized in this study stained a pale blue color indicating that the mucins of the urinary bladders in this study were all of the acidic variety. Since all of the mucins visualized in this study were of the same variety with regard to pH regardless of treatment group no statistical analysis was conducted in relation to treatment effect on the type of mucin.

Mucin Thickness:

Ten bladders from BL mice were observed in this study. Representative samples of this tissue can be seen in Figures 2 and 3. The calculated average thickness of the mucin layer across all 10 BL bladders was 6.725 μm. The individual averages for each baseline bladder can be seen in Table 1. Ten GC bladders were used in this study. Representative samples of this tissue can be seen in Figures 4 and 5. Upon observation of the tissue samples, it was found that GC bladder 37 displayed abnormal morphology compared to the other 29 bladders in this study, therefore this bladder was removed from the study. The calculated average thickness of the mucin layer across the 9 remaining GC bladders was 6.305 μm. Table 2 shows the average mucin layer thickness for each individual GC bladder. Ten bladders from FL mice were observed in this study. Representative samples of this tissue can be seen in Figure 6 and 7. When analyzing the FL tissue sections, it was noticed that FL bladder 2 appeared to not be bladder tissue. As previously stated, the bladders were part of tissue sharing program, and this tissue could be vaginal or intestinal tissue, so it was removed from the study. The calculated average thickness of the mucin layer across the 9 remaining FL bladders was 5.836 μm. The individual averages for each FL bladder can be seen in Table 3.

A statistical comparison of the individual mucin averages from each of the three treatment groups of bladders was conducted via a one way ANOVA using the statistical software Minitab 16 (Table 4). This allowed for comparisons to be made between the three groups. Using a 95% confidence interval, the results showed a statistical difference in the thickness of the mucin layer between BL and FL urinary bladders ($p=0.022$). No statistical difference was found when comparing GC to FL ($p=0.168$), or BL to GC ($p=0.276$).

Discussion:

The results of the ANOVA show statistical significance between the BL and FL mucin thickness. Based on these results we can conclude that spaceflight has an effect on the production of mucin in the urinary bladder. Based on the information in Table 5, we can conclude that this was an inhibitory effect. There was no statistical significance when BL and GC tissues were compared. This is expected because both of these control groups were housed at the Kennedy Space Center. When GC was compared to FL tissues, there was no statistical significance. This result was somewhat puzzling. This raises the question why one control group is different from the flight tissue and the other is not.

The BL mice were housed in regular rodent cages. These cages would have had bedding in which the mice could bed down. These cages would also have the water in unpressurized bottles, and have food in a regular bowl. The CBTM-2 cages, which were built for the mice to survive in space, had an altered environment. The CBTM-2 cages did not contain bedding for the mice to bed down, and the sides of the cage were lined with rodent chow. Thus, the mice housed in the GC CBTM-2 cages were living in a different environment than their BL counterparts. Was this change in environment enough to cause slight changes in the mouse physiology? Could these changes slightly decrease the thickness of the mucin layer in the urinary bladder? That would be the subject of a new study. The flight mice had to not only learn to live in a new cage, but also had to learn to eat, drink, and sleep while floating in a microgravity environment. This could have had even more of an impact on the physiology of the mice than that seen in the BL animals.

In conclusion, this preliminary study allowed for qualitative and quantitative analysis of mucin layer thickness in the urinary bladder. The results of this study indicate that all three treatment groups had mucins in the same pH range. We therefore conclude that spaceflight does not have an effect on the pH of the mucin layer of the urinary bladder. The results also indicate that the mucin layer of the urinary bladder in FL mice is significantly thinner than that of BL mice. However, no statistical difference was seen between BL and GC mice or between GC and FL mice. There appears to be a trend toward thinning of the mucin layer in the urinary bladder as we progress from BL to GC to FL mice. This is an indication that some factor of spaceflight is responsible for this thinner mucin layer. Presumably this factor is the exposure to microgravity. However, during spaceflight animals are exposed to high levels of cosmic radiation. Since no mechanism was set in place to control for this radiation, it cannot be ruled out as a, if not the, causative factor. Based on non-significant differences between BL and GC and GC and FL mucin thicknesses, we conclude that the cage environment also plays a role in this difference.

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Figures & Tables

Figure 1: Ground Control Mice in CBTM-2 cage.

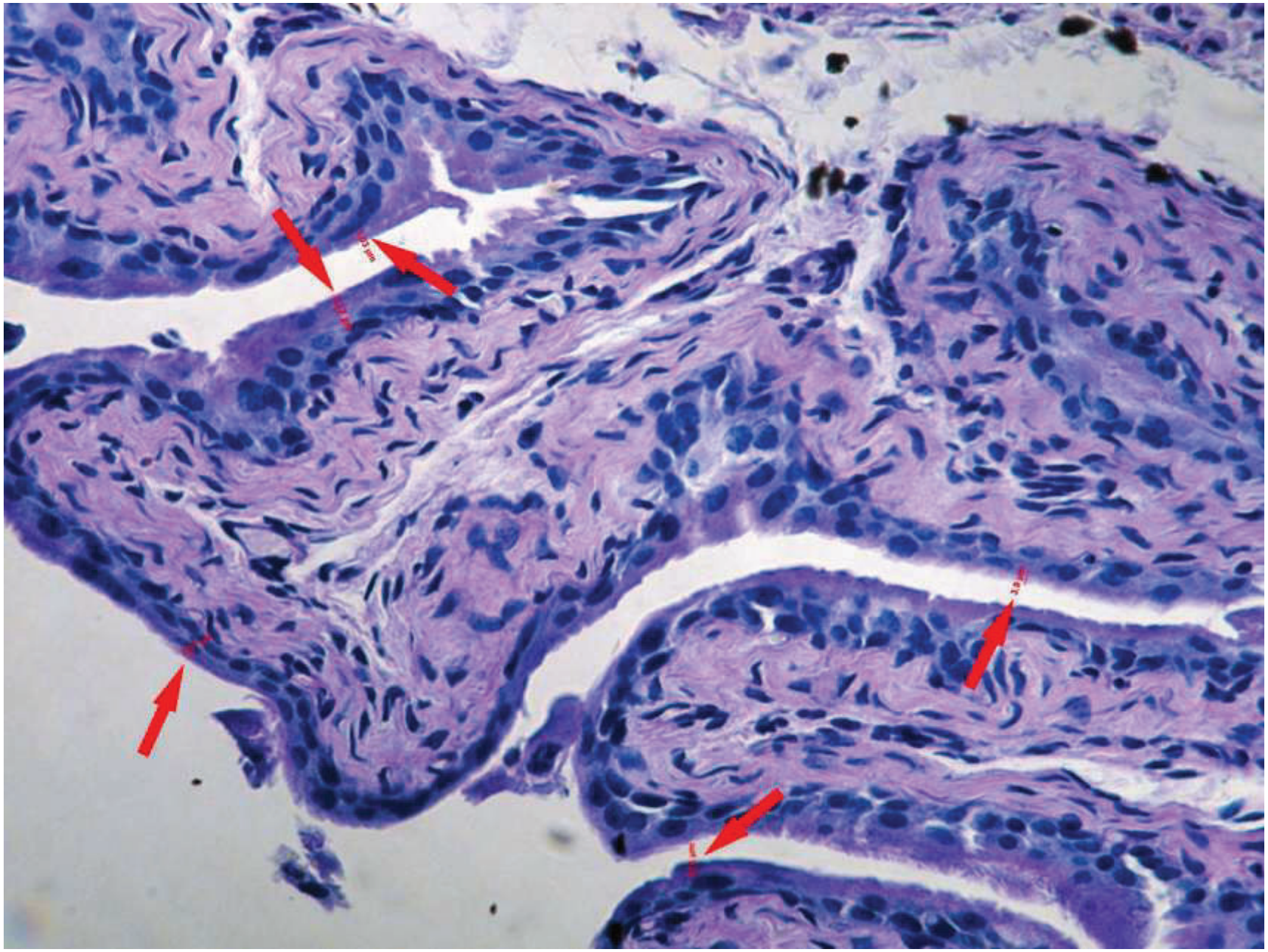

Figure 2: Photomicrograph of Baseline urinary bladder (X400). Note the folds of the bladder wall (rugae). Arrows = regions where mucin thickness was measured.

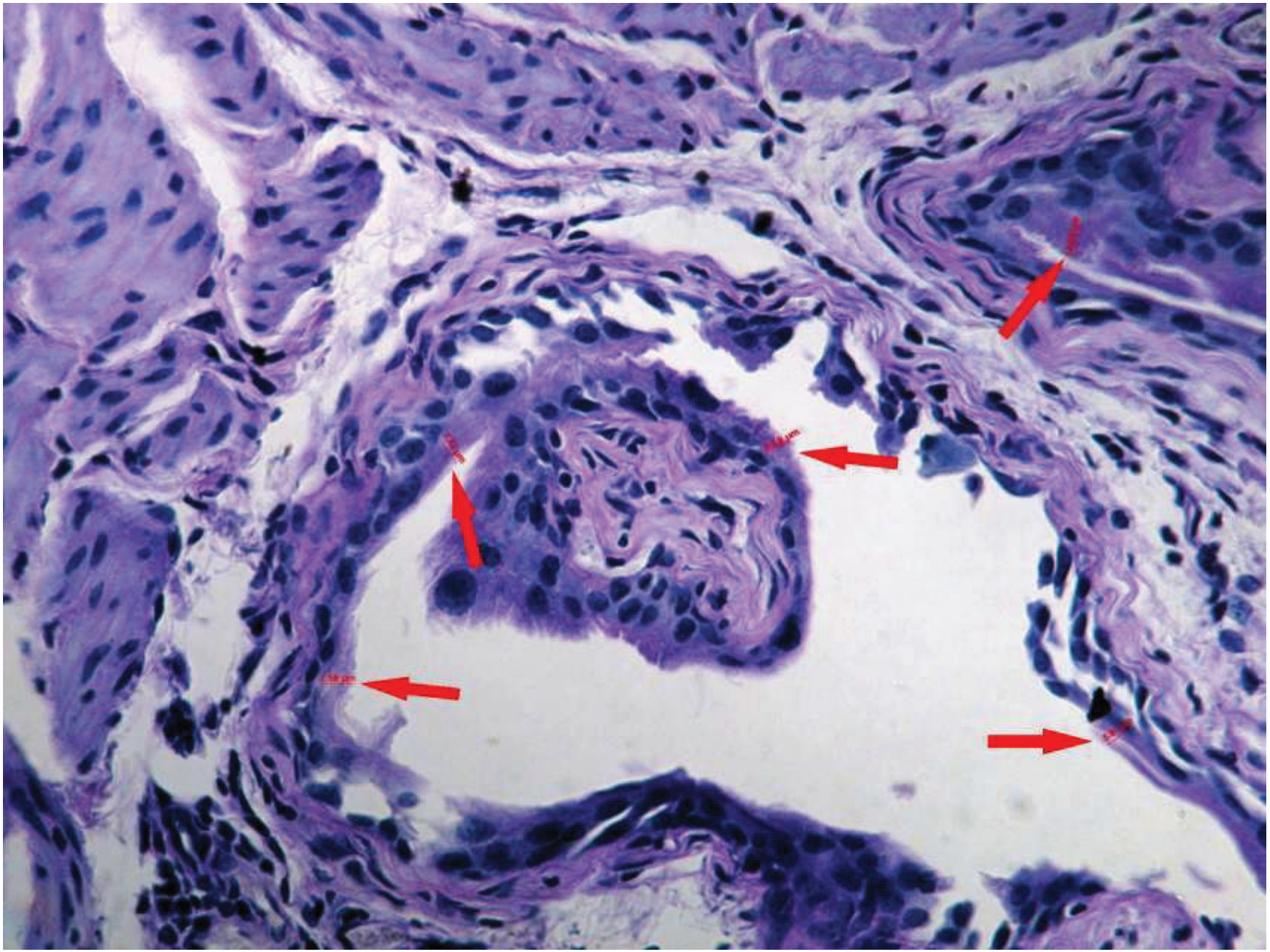

Figure 3: Photomicrograph of Baseline urinary bladder (X400). Arrows = regions where mucin thickness was measured.

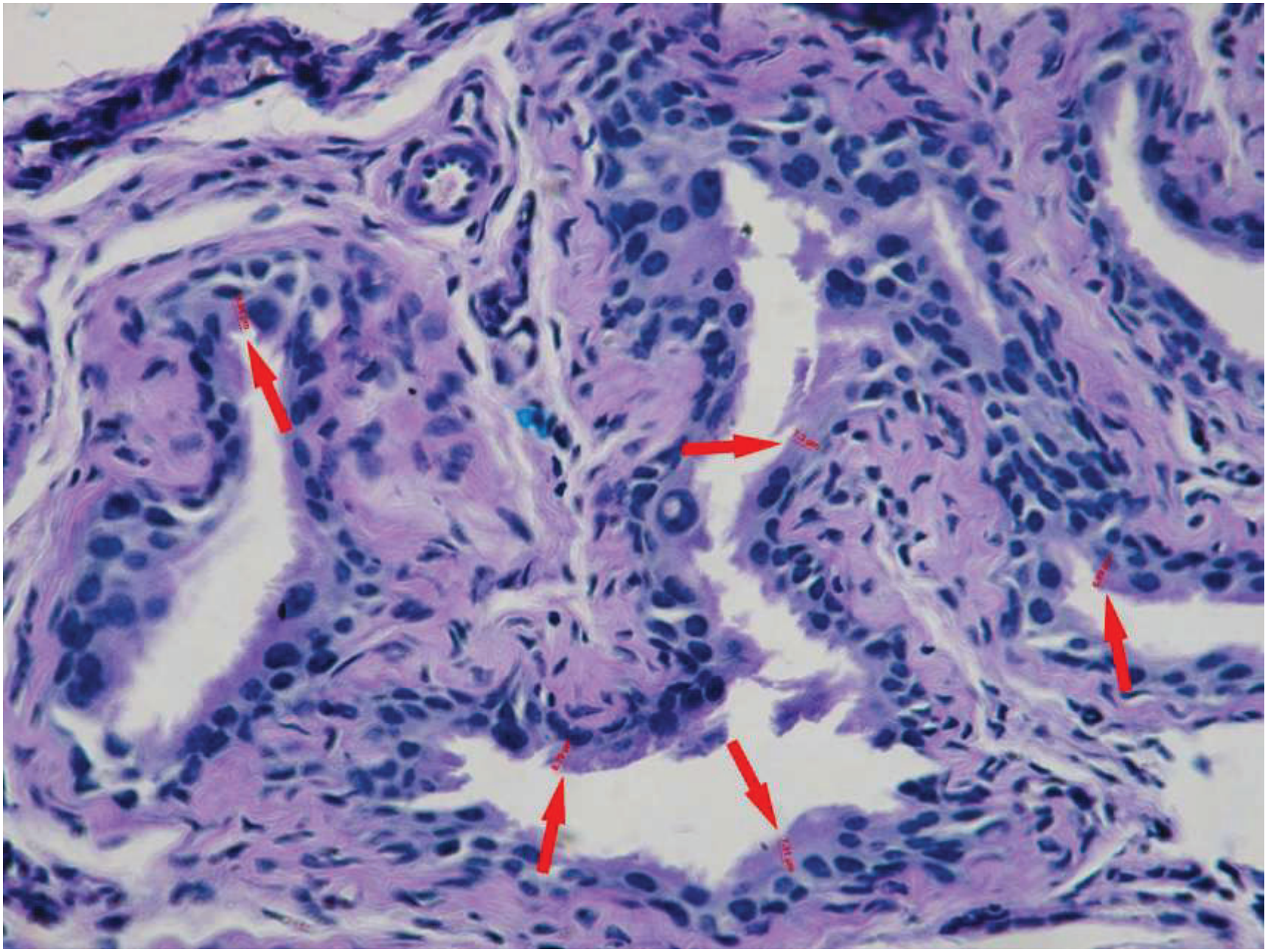

Figure 4: Photomicrograph of Ground Control urinary bladder (X400). Arrows = regions where mucin thickness was measured.

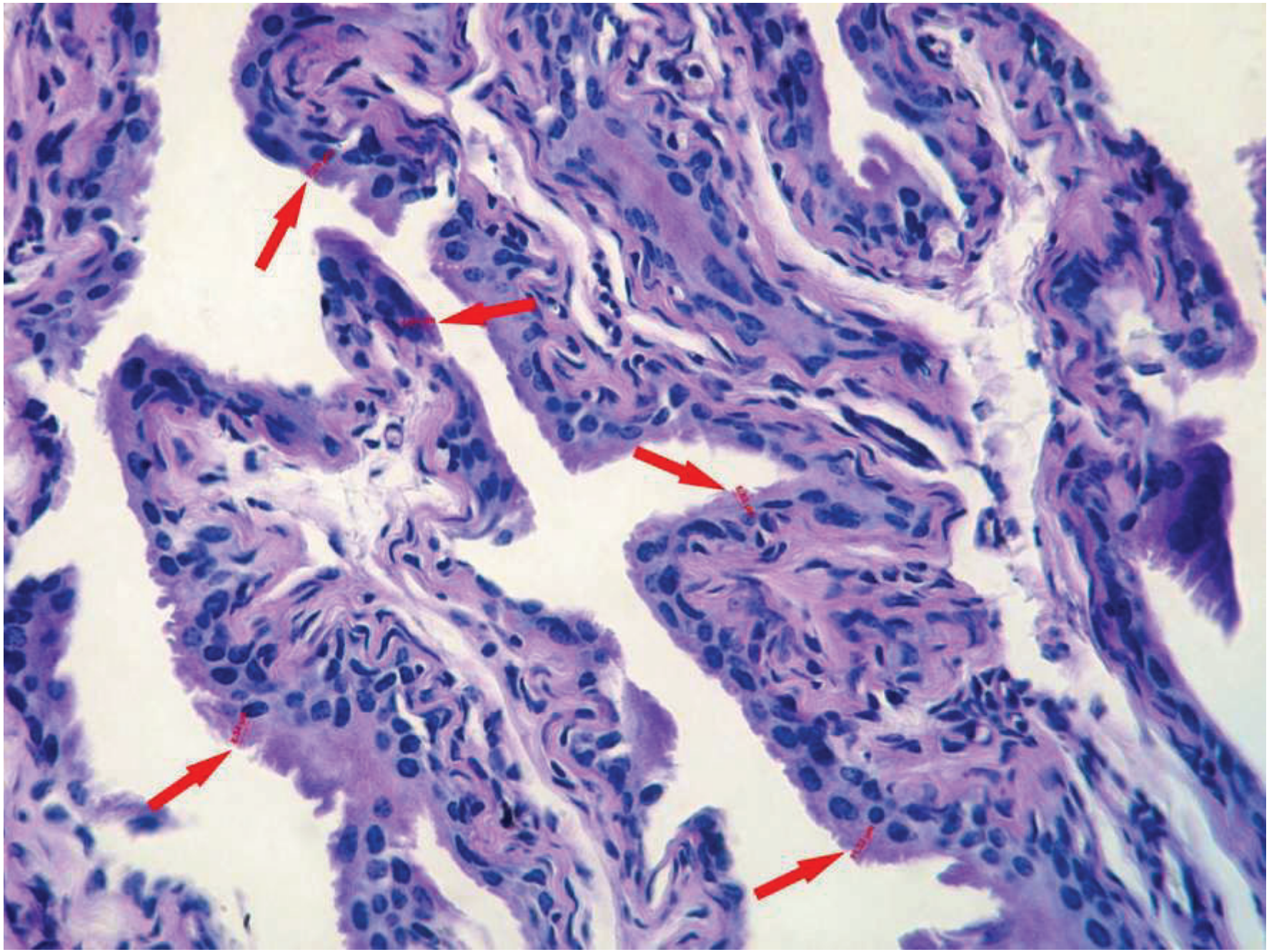

Figure 5: Photomicrograph of Ground Control urinary bladder (X400). Arrows = regions where mucin thickness was measured.

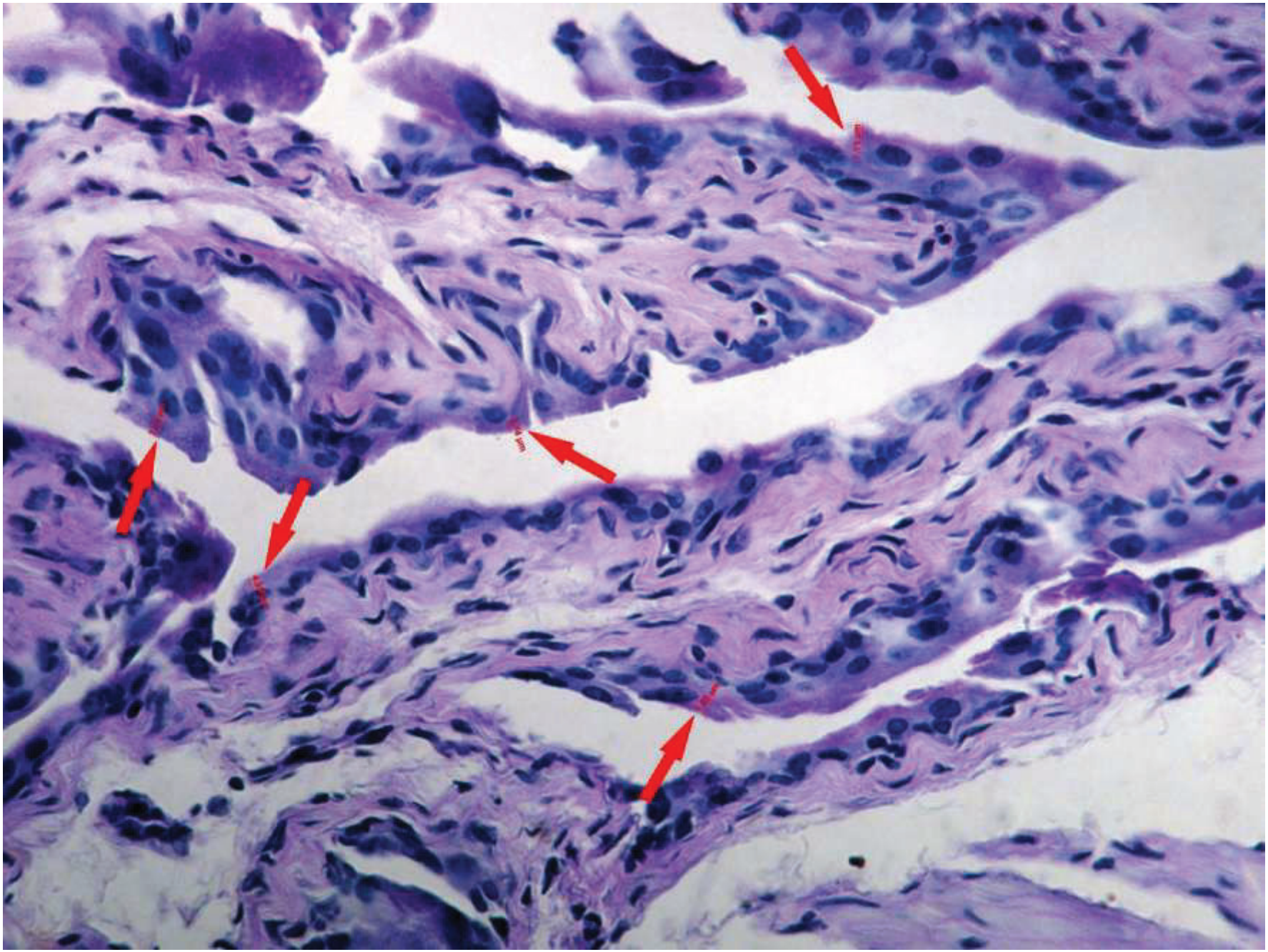

Figure 6: Photomicrograph of Flight urinary bladder (X400). Arrows = regions where mucin thickness was measured.

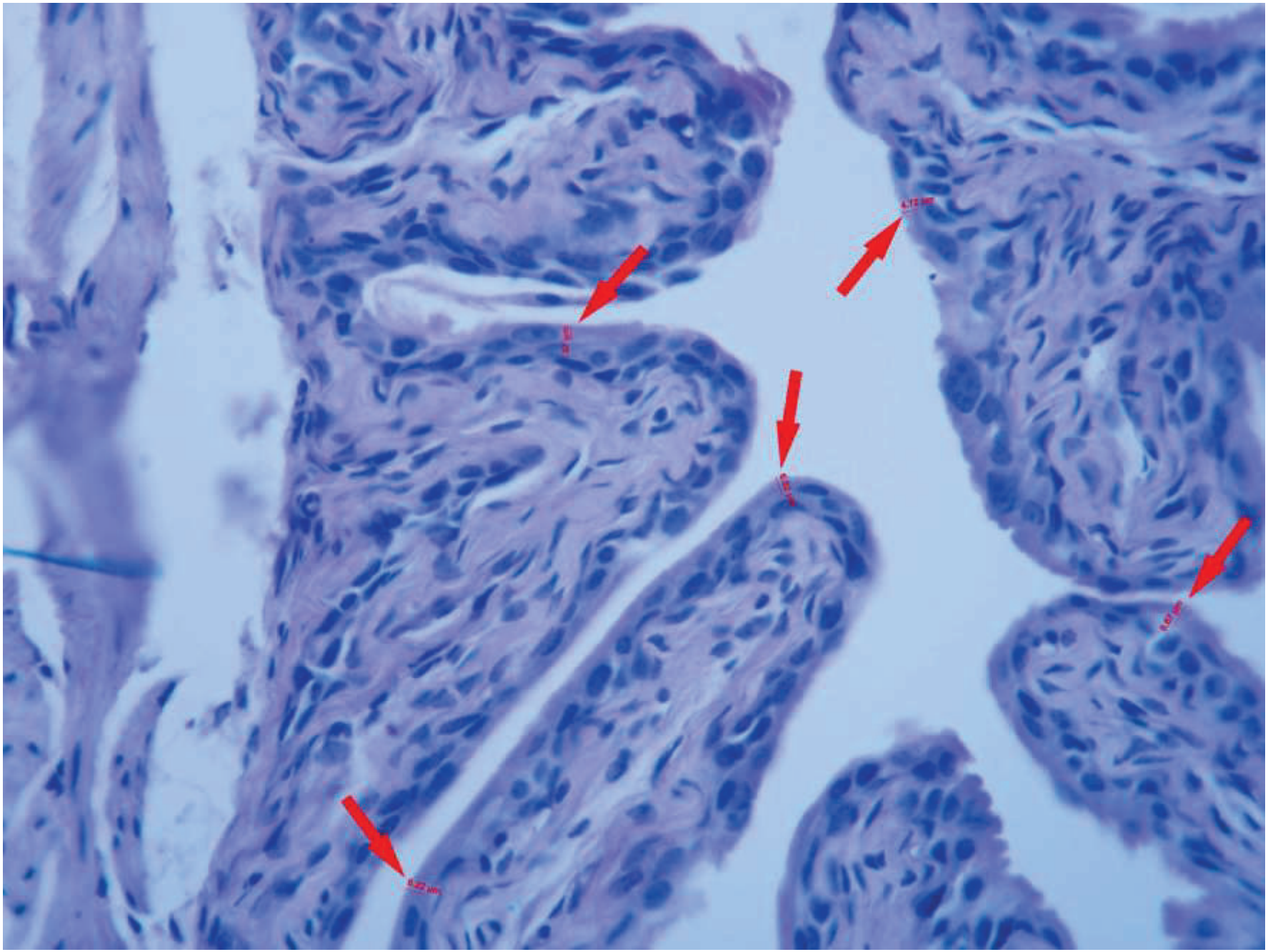

Figure 7: Photomicrograph of Flight urinary bladder (X400). Arrows = regions where mucin thickness was measured.

Baseline Bladder Average Mucin Layer Thickness	
Bladder Tissue	**Average Thickness (µm)**
BL 61	7.928
BL 63	5.993
BL 65	5.418
BL 66	6.187
BL 67	6.577
BL 68	6.577
BL 69	6.455
BL 70	6.665
BL 71	8.341
BL72	7.114

Table 1: Average Mucin Thickness for Baseline Bladders.

Ground Control Average Mucin Layer Thickness	
Bladder Tissue	**Average Thickness (µm)**
GC 32	5.713
GC 33	7.782
GC 38	6.755
GC 43	5.848
GC 46	5.869
GC 50	6.339
GC 51	6.945
GC 52	6.067
GC 53	5.429

Table 2: Average Mucin Thickness for Ground Control Bladders.

Flight Average Mucin Layer Thickness	
Bladder Tissue	**Average Thickness (µm)**
FL 5	5.989
FL 8	6.147
FL 10	6.091
FL 12	4.859
FL 15	6.886
FL 16	6.132
FL 20	6.047
FL 21	5.152
FL 24	5.221

Table 3: Average Mucin Thickness for Flight Bladders.

ANOVA Results			
	Baseline vs. Ground Control	Ground Control vs. Flight	Baseline vs. Flight
p value	0.276	0.168	0.022

Table 4: p values from the one way ANOVA comparing Baseline, Ground Control, and Flight tissues, based on a 95% confidence interval, p=0.05.

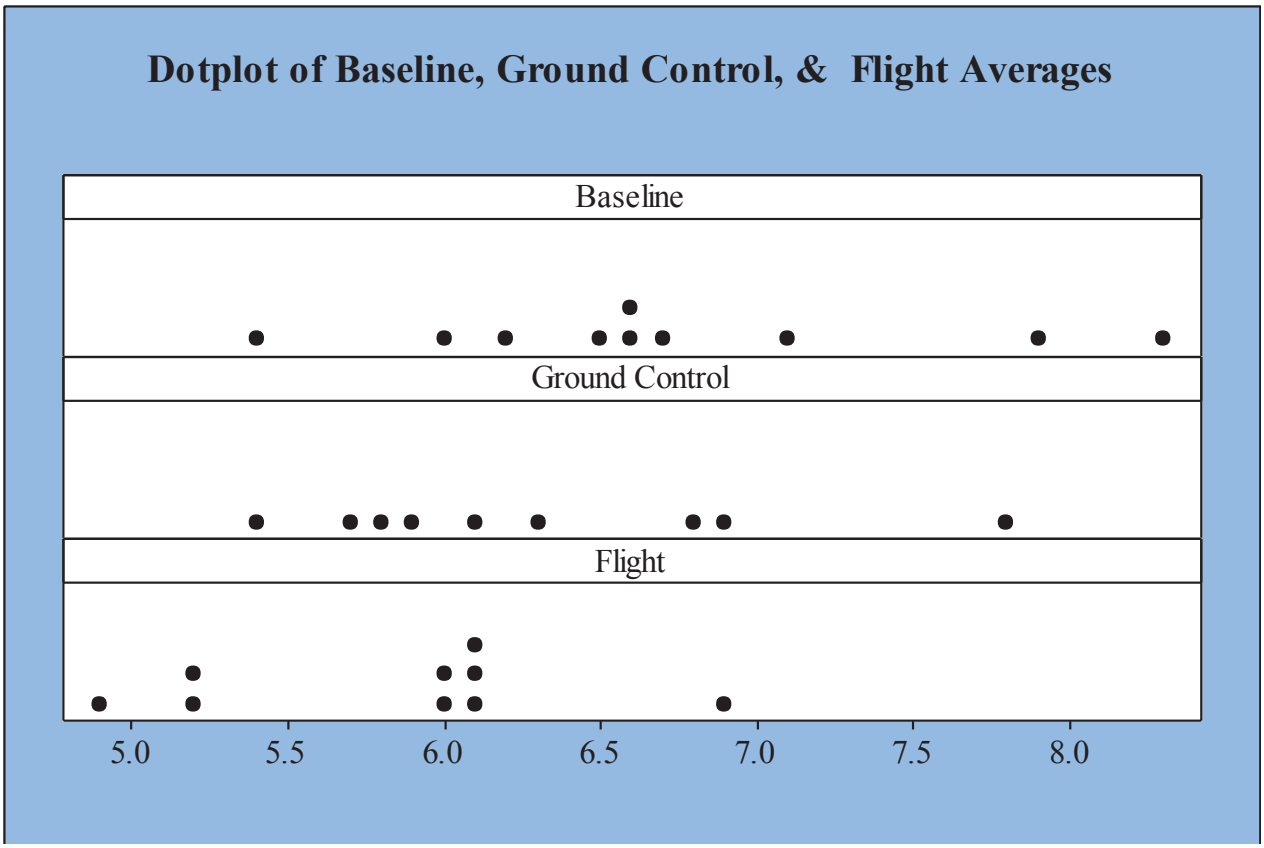

Table 5: Dotplot of average mucin thickness.

Acknowledgements

Dr. Allan Forsman, Thesis Advisor

Dr. Laraine Powers, Faculty Reader

Dr. Michele Crumley, Faculty Reader

Dr. Michael Cody, Midway Honors Director

ETSU Department of Health Sciences

ETSU Honors College

Amgen Corp (Thousands Oak, CA), for principal funding of the study

BioServe Space Technologies (Boulder, CO), for design and use of CBTM-2 cages.

ETSU Small RDC Grant #11-022sm