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TIMED ACID BATH EFFECTS ON KIDNEY PODOCYTES PREPARED FOR SCANNING ELECTRON MICROSCOPY

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Abstract

In perfusion fixation of dog kidney glomeruli, observation of tear fractured surfaces by scanning electron microscopy reveals the deposition of "debris" on the surface of glomerular visceral epithelium (podocytes) that is not deposited on other kidney tissue surfaces. It is of concern to remove this debris without damage to tissue surfaces. A 5 minute bath in 8N HCl (hydrochloric acid) following postfixation in 1% OsO_4 (osmium tetroxide) removes only some of the debris. A 10 minute acid bath removes this debris without visible damage to podocyte surface structures such as cilia, plicae or blebs. A 20 minute acid bath is destructive to these surface features.

Key words: acid bath, scanning, scanning electron microscopy, hydrochloric acid, kidney, podocytes, cilia, surface blebs, glomerulus, dog.

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Introduction

Several chemical and enzymatic techniques have been employed to remove the extracellular material from cell surfaces in order to obtain unobscured SEM (scanning electron microscopic) views of cell surfaces. However, Shimada (1981) cautioned that in every case where this is to be done, trials should be performed to determine the best techniques for the removal of extracellular material from any given tissue.

Evan *et al.* (1976) have used a combination of HCl (hydrochloric acid) and collagenase to remove both the connective tissue and the basement membrane. This technique exposes the roughened cell surface which interdigitates with the basement membrane in skin, kidney and autonomic ganglia. Anderson *et al.* (1988) used HCl without OsO_4 (osmium tetroxide) postfixation on renal microvasculature of the black bear while Shimada (1981) reported using HCl and collagenase treatment prior to postfixation (with OsO_4) of monkey thyroid tissue.

Krizmanich and Lee (1987) reviewed four techniques of tissue preparation: 1) KOH with collagenase, 2) HCl with elastase, 3) HCl with collagenase, and 4) postfixation with 2% OsO_4 followed by HCl treatment. They found that the fourth technique was the best method for removal of extracellular, adventitial matrix from large mesenteric arteries in rats, suggesting that pretreatment by OsO_4 allowed for less time in the subsequent acid bath thus reducing the damage to cell surfaces. The temperature and time within the HCl bath is important (Krizmanich and Lee, 1987; Anderson *et al.* 1988; Shimada, 1981; Evan *et al.* 1976 and Evan *et al.* 1984) whenever chemical and/or enzymatic digestive techniques are used in the preparation of tissues for subsequent SEM study.

As part of an ongoing study, we are studying the kidney glomerular tissue and vasculature using corroded and non-corroded tissue casts. Initial preparations of the kidneys from three dogs revealed a pattern of deposition of debris which interfered with the examination of the visceral epithelium of the glomerulus. Debris was deposited on the surface of the visceral epithelium but was not seen on other surfaces. In an attempt to remove this

debris, the method of Krizmanich and Lee (1987), in which OsO_4 postfixation followed by a 20-25 minute bath in 8N HCl at 60°C, was used to prepare the non-corroded tissue for SEM study. This method was chosen over those which used various enzymes because a basement membrane is not present on the cell surfaces being studied. When applying the technique described by Krizmanich and Lee (1987), our preliminary SEM observations of kidney glomeruli suggested that the recommended time of 20-25 minutes of acid treatment caused excessive tissue damage and a loss of potentially important cell surface details. Because of these observations, the present qualitative study was performed to examine the time-related effects of acid treatment on the removal of debris from the visceral epithelial cell surfaces of the kidney glomerulus without damage to surface structures of these cells. Our intent was to effectively remove the debris consistently seen deposited upon glomerular visceral surfaces in order to qualitatively improve the observation of these surfaces without damage to the tissue surfaces.

Materials and Methods

A female dog weighing approximately 30 pounds, obtained from the Office of Laboratory Animal Care, College of Veterinary Medicine, was anesthetized by an intravenous injection of sodium pentobarbital to effect. As part of another experiment, the animal had been surgically prepared to measure blood flow by implantation of small thermistor probes into the liver and one kidney. After that experiment was completed, the animal was euthanized with an overdose of sodium pentobarbital. The other kidney was removed, and the renal artery cannulated. The kidney was then perfused with a modified Hank's solution containing 4% (w/v) mannitol to flush the blood from the kidney vasculature. This was followed with approximately 60 ml of 5% buffered formalin. At the same time, Mercox resin (see Lametschwandtner *et al.* 1990) was prepared and was hand-injected into the kidney vasculature via the renal cannula at the termination of the formalin flush. The Mercox was allowed to polymerize for one hour at room temperature with the kidney wrapped in plastic wrap. Then the kidney still wrapped was placed in a refrigerator for 24 hours. Small sections of the cortex were cut away with an acetone cleaned razor blade, rinsed in buffer and immersed into 2.5% glutaraldehyde in 0.1 M cacodylate buffer in 3.5% sucrose. This method of processing both the tissue and cast (non-corroded casting) follows that of Castenholz (1983). The technique allows for greater support of vessels by the polymerized cast during processing and is believed to help maintain spatial relationships during subsequent drying procedures (Castenholz 1983).

Tissue sections were subsequently removed, rinsed in buffer and torn apart manually (fingers were cleaned to remove oils and particulate matter) in a manner similar to that reported by Lemley *et al.* (1986) and Lipsett *et al.* (1987). The resulting tear fractured pieces were reimmersed in fixative for a period of one hour. Tissue pieces were trimmed to a block approximately 1cm X 1cm X 0.25cm, rinsed in buffer and postfixed in 1% OsO_4 for one hour, and washed in distilled water. Randomly selected pieces were placed in warmed (60°C) 8N HCl solution for either 5, 10 or 20 minutes. Control samples were placed in distilled water for no more than 20 minutes.

At the end of the respective time in the acid bath, the samples were washed in distilled water (3 X 10 min, 300 ml volume). All samples were then placed into small polyurethane cups with just enough distilled water to cover the surfaces of the blocks and frozen overnight at -20°C, after which they were freeze dried in a LABCONCO MODEL 5 freeze dryer. This method of freeze drying was chosen not only because of less shrinkage to the cast than in critical point drying (Lametschwandtner *et al.* 1990) but also because alcohol or acetone dehydration would soften and distort the cast (Murakami *et al.* 1971).

The dried samples were mounted tear fractured surface up (as readily determined by examination under a dissecting microscope) on 1-1/8 inch (2.85 cm) stubs (Ted Pella, Inc.) and sputter coated in the SPI sputter-coater for viewing in the ISI-40 scanning electron microscope. It is relevant to note that the pieces of non-corroded vascular casts can be corroded at a later time for future investigations of the vascular casts.

All tissue blocks of all treatments and controls were examined with the scanning electron microscope. Each block contained several glomeruli where Bowman's Capsule had been torn and the visceral surfaces exposed, while other glomeruli were observed with their Bowman's Capsule intact.

Results and Discussion

Kidney tissue receiving the control treatment is shown in Figure 1. A considerable amount of extracellular debris was typically present in the urinary space of all glomeruli using the control method of tissue preparation. This observation was consistent with all dog kidney preparations we had previously attempted with or without, vascular casting. Observations suggested that this debris results from the precipitation of urine components during the fixation or drying process because this debris is only found on tissue exposed to urine. That is, tissue surfaces usually exposed to urine showed the debris while tissue surfaces not normally exposed to urine were free of the debris.

The ten minute acid treatment removes most debris and provides an unobstructed view of surface details of the podocyte as shown in Figures 2 and 3. Many cell surface microprojections or blebs similar to those described by Andrews (1975), are clearly visible on the podocyte and its major processes (Fig. 2) even at low magnification. In Figures 4 and 5, the cell surfaces of the dog kidney podocytes treated in 5 and 10 minute acid

baths, respectively, show very prominent microprojections, blebs and folds of all shapes and sizes. The overall surface appearance of these cells is extremely roughened.

Figure 4 shows surface structures of the cell body which are similar to those described by Andrews (1975 and 1988) as "rudimentary" or "primary" cilia. He found that cilia of this type are of variable lengths, and frequently arise from the middle of the podocyte cell body in both human and rhesus monkey kidneys. Using tissue cultures of thinly sliced rat kidney, Andrews (1981) observed that microfolds or plicae may form in the cell surface. These structures may then detach at one end to form slender finger-like microvilli. Figure 5 shows the variety of blebs, folds and microprojections found in the dog.

As can be seen in Figure 5, a long finger-like projection appears to run parallel to the long axis of the podocyte cell body and appears attached at both ends and possibly along its middle portion. Shorter projections may also be seen in the central part of the cell body. Another projection is seen to project away from the cell body but its termination is uncertain.

A podocyte that has been treated for 20 minutes in the acid bath is shown in Figure 6. When compared to the surfaces of cells treated for shorter times (Figures 4 and 5), it appears that the extra time in the acid bath has etched away some portion of the podocyte cell surface. Although the surface is still roughened, the prominent microprojections, such as those evident in Figures 4 and 5 are no longer visible.

Pedicels are well demarcated in the control tissue (Fig. 1), the 5 minute (Fig. 7) and 10 minute (Figs. 2 and 3) acid treated tissues. At higher magnifications, surface structure of podocyte processes and the pedicels are readily distinguishable after 5 and 10 minute acid treatments as shown in Figures 7 and 3.

Figures 8 and 9 show the results of a 20 minute acid bath on podocyte cells and their processes. Compared to the control and the 5 and 10 minute acid treatments, the pedicels of tissue treated for 20 minutes appear to have been etched away and their structure is no longer well defined. The podocyte cell body shown in Figure 8 also appears to have been disrupted.

Since this experiment, the 10 minute acid bath treatment has been applied to two other dog kidney preparations with results consistent with experimental results. That is, very little debris was observed upon glomerular epithelial surfaces nor was any discernible damage observed.

Conclusion

Data presented here suggest that cell surface structures may be distorted or destroyed when tissues are exposed to an 8N HCl solution for periods of time in excess of approximately 10 minutes. An acid bath treatment for 20 minutes was found to cause the destruction of kidney podocyte cell membrane structures. Data support the suggestion that a 10 minute bath is sufficient to rid the cell surfaces of essentially all of the debris present on the podocyte surface while also sparing the surface from the destructive effects observed with a 20 minute treatment. In our experience, the 10 minute bath was most effective because the 5 minute exposure did not uniformly remove the debris from the visceral epithelial surface.

Acknowledgments

Thanks are extended to the Center for Electron Microscopy of the University of Illinois, for the use of the scanning electron microscope and to the Electron Microscope Suite of the College of Veterinary Medicine, also of the University of Illinois, for the use of the darkroom facilities. We wish to thank Lisa Xuemin Xu and Kathy Lin for their help in our laboratory and to J. Patrick Wynes and Patrick Porter of the Anatomy Preparation Laboratory of the College of Veterinary Medicine for their assistance in the freeze drying process. This study was supported by NIH grant NHLBI-HL27011.

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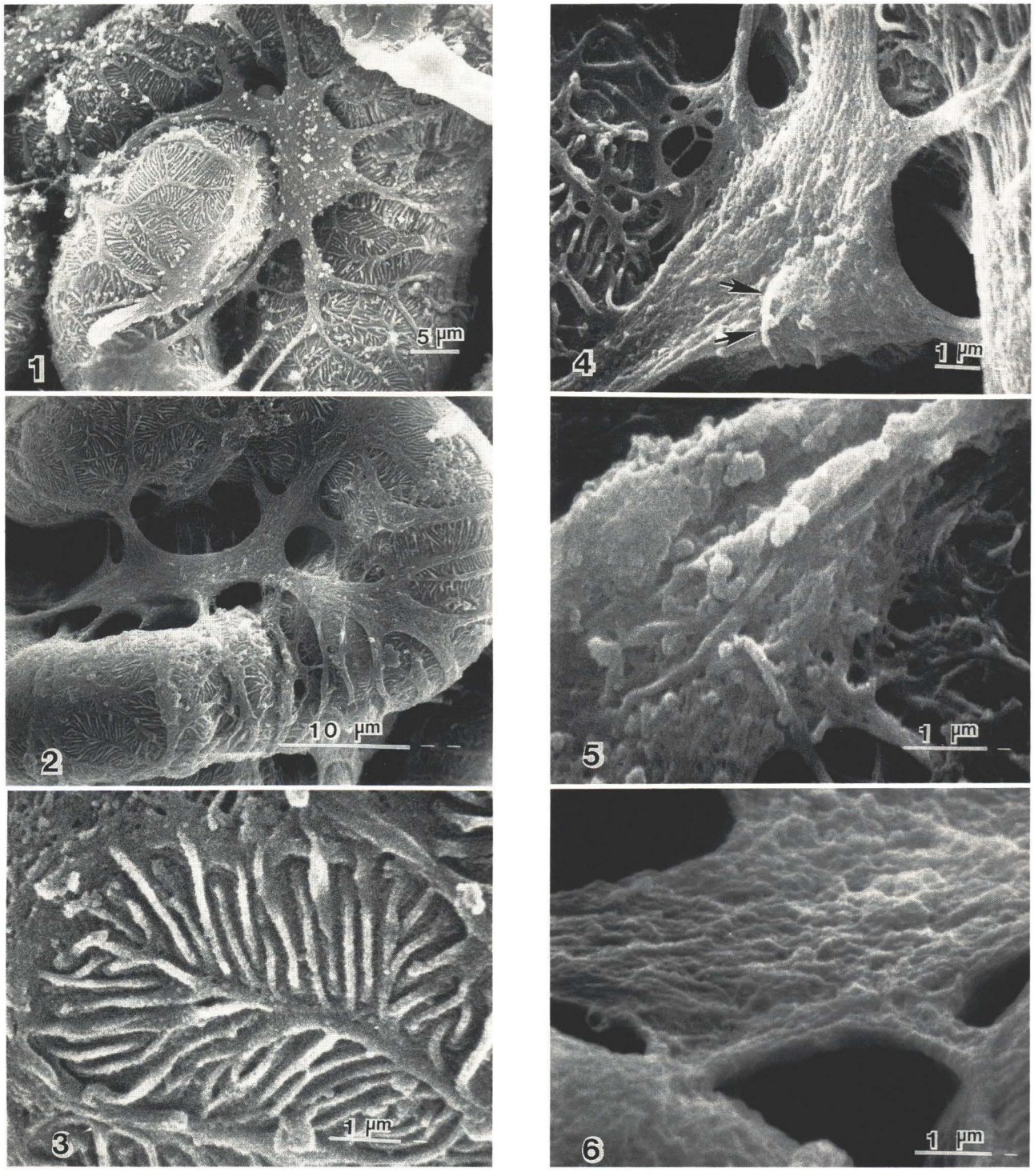
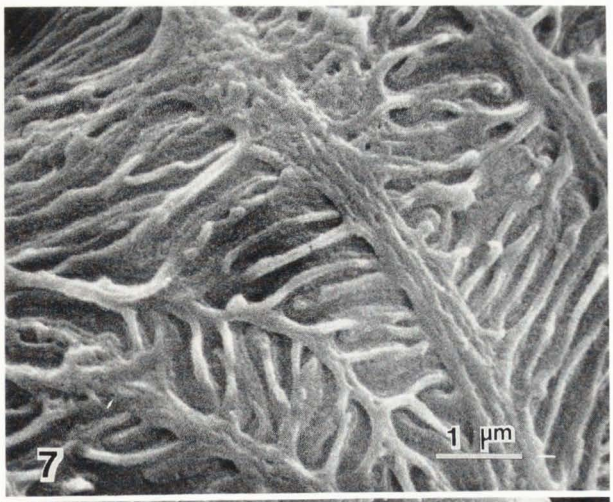
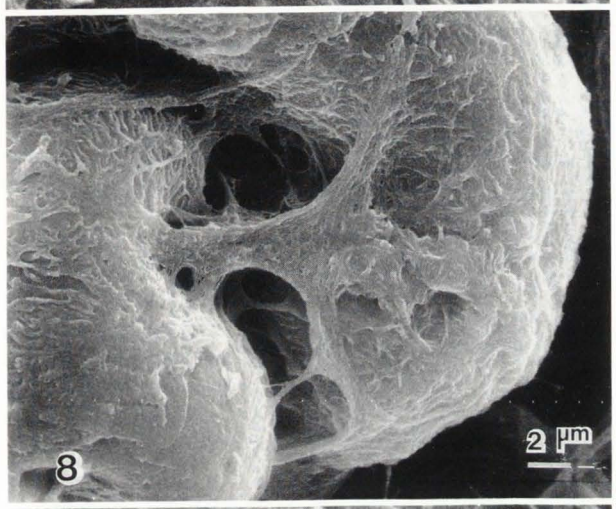


Fig. 1: Dog kidney glomerulus. No acid treatment. **Fig. 2**: Dog kidney glomerulus following a 10 minute acid bath treatment. **Fig. 3**: Podocyte and interdigitating pedicels after a 10 minute acid bath. **Fig. 4**: Note the long cilia-like structure (arrow, identified based on Andrews 1975, 1988) near the middle of the cell body. A 5 minute bath. **Fig. 5**: Several elongated folds and numerous blebs are obvious (10 minute bath). **Fig. 6**: Even though the surface is still roughened, folds and blebs appear to have been etched away after a 20 minute bath.





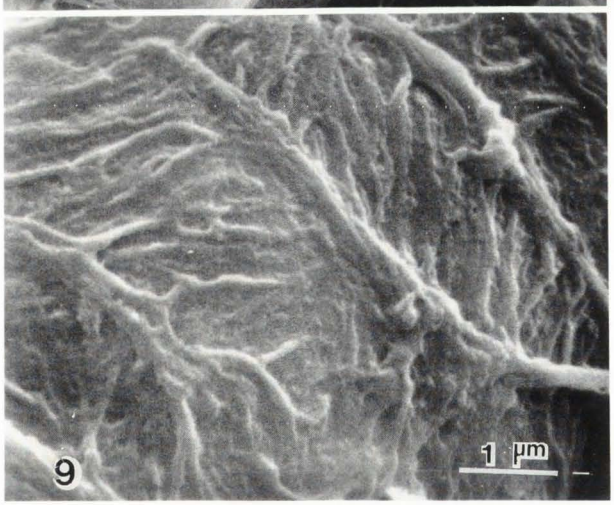


Fig. 7: Podocyte process with interdigitating pedicels. Dog kidney glomerulus after a 5 minute acid bath.
Fig. 8: Dog kidney glomerulus following a 20 minute acid bath.
Fig. 9: Dog kidney podocytes interdigitating pedicels have been severely damaged after a 20 minute acid bath.

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Discussion with Reviewers

D. Casellas: Have the authors observed "debris" at the surface of other structures exposed to urine e.g., Bowman's Capsule or proximal tubules?
Authors: Yes "debris" was seen as frequently on the parietal surface of Bowman's capsule as on the glomerular surfaces. This observation was part of our assumption that the material was due to precipitation of material in urine. Unfortunately very few luminal surfaces of tubules were observed, not enough for us to make a generalization. We would also like to comment that the outer surface of Bowman's capsule was always very clean and free of the "debris" noted on the internal surfaces of the capsule.

D. Casellas: Could the authors comment on the effects of 10 minute treatment on other fragile surface structures e.g. proximal brush borders.
Authors: As stated above, very few luminal surfaces of tubules were observed. Also, because of our research interests, we were principally concerned with glomerular surfaces. Thus we can not comment on the effect upon tubule luminal surfaces.

Reviewer IV: I am not convinced by the argument that the source of the debris was urine. Similar type of debris is often found in fractured specimens. It is a mystery to me that these authors would use acetone-cleaned razor blades to cut small sections of the cortex, and then used their fingers (previously cleaned with acetone?) to fracture the tissues. Therefore there could be many sources for the debris.
Authors: As stated in our text, the "debris" was specifically located over areas normally exposed to urine within the glomerular system. This "debris" was not located over any other tissue surfaces. We agree that the only way to answer this question is with the analysis of the "debris". We only suggested the possible source of the

"debris" based upon the observed evidence of our study.

In reference to razor cutting small sections (reviewers' emphasis), the sections were cut down through the depth of the cortex wide enough and long enough for easy manipulation. We were fully aware of the possibility of introducing contaminants such as oils, etc., and took precautions to reduce the problem by cleaning both razor blades and hands thoroughly.

Additional information on manual methods to produce a tear fracture can be found in: Andrews (1981), Vonnahme and Muller (1981), Macchiarelli and Motta (1986), Andrews and Porter (1974), and Grisham *et al.* (1975).

There are always sources of debris to contend with but when proper procedures for tissue preparation of SEM samples are followed and the "debris" consistently appears only on glomerular visceral surfaces and not other surfaces, then precipitates of urine during fixation becomes the most likely possibility. The reason this study was undertaken was that this phenomenon occurred with every dog whose kidney we prepared for SEM. This was not an observation of just one dog.

Reviewer IV: The authors have stated that four blocks of tissues were processed for each time in acid, but did not say whether all the tissues were examined with SEM. They also stated that since then, this technique has been utilized with other tissue samples with consistent results, but did not say what kind of tissues were used, and explain what they meant by "consistent results". Four samples from one dog, with the observations done probably only once (?) on one (?) sample from each group does not really give one any confidence about the results. To be acceptable, they should carry out more studies with samples from more dogs, and provide quantitative data to substantiate their statements. In addition, they should (a) Provide SEM micrographs at the same magnification for different treatment groups for comparison, (b) Provide quantitative analysis to show the difference among the different groups methods.

Authors: We routinely examine all available tissue before making final judgements about what we find. Differences between the blocks per treatment would have been reported.

All glomeruli seen in each block per treatment was examined. Where Bowman's capsule was torn away, "debris" was found only on the visceral epithelium in all cases. All glomeruli observed per block showed "debris" present, "debris" was not present on any other tissue surface. Where glomeruli were still encased in their capsules, no debris was observed on the outside of the capsule.

Following the results of this study, other dog kidneys were prepared based upon this study. A 10 minute acid bath in each case resulted in the same results as described here.

This study was undertaken for qualitative, and not quantitative, purposes. The debris observed in all previous preparations of dog kidney for SEM interfered with detail observations of kidney visceral glomerular surfaces in the dog. This problem was encountered in each dog kidney processed for SEM. We were thus concerned with improving the quality of preparation in order to improve the quality of observations and thus to improve collection of quantitative data in subsequent studies. The problem was not an isolated one and the results of our procedure, when applied to subsequent dog kidney preparations for SEM, improved the quality of observations due to the absence of debris on visceral surfaces.

Reviewer IV: I am not convinced that the structure described in the text, and shown in Figure 4 are cilia.

Authors: Our designation of certain structures as cilia or cilia-like is based upon micrographs and descriptions of rudimentary cilia of podocytes described in the papers cited in the text and in figure caption.

Reviewer IV: Artifacts due to freezing are still a major concern, which will vary depending on the size of the specimen, because this would affect the rate of freezing of the tissues. Therefore, even though the tissues were frozen and processed at the same time, there is no guarantee that the rate of freezing of these tissues were the same.

Authors: All tissue blocks were of nearly equal size. We agree that there are possible artifacts formed as a result of any and all methods of tissue processing. The point of this paper is that debris was selective in its deposition upon visceral epithelial surfaces of glomeruli of the dog. It is our contention however, that artifacts in the process of freezing would not selectively place debris in the very precise, and selective pattern observed.

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