Scanning Microscopy

Volume 1 | Number 3 Article 47

4-6-1987

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Scanning Microscopy, Vol. 1, No. 3, 1987 (Pages 1339-1347) 0891-7035/87$3.00+.00
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

SCANNING ELECTRON MICROSCOPY OF VASCULAR CORROSION CASTS IN COMPARATIVE STUDIES ON RENAL VASCULAR STRUCTURE

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(Received for publication January 16, 1987, and in revised form April 06, 1987)

Abstract

In comparative microcorrosion casting studies on renal vascular systems the following demands should be met: The preparation procedure (anaesthesia, operation, flushing of the blood vascular system, ...) should be in accord with the specific physiological properties of the animal under investigation and the casting procedure (injection, curing, maceration, ...) should be kept constant as far as possible. If these points are considered, comparative data, even of quantitative nature, can be obtained from corrosion casts. Examples of results at the organ, single vessel and intercellular level as well as correlation of the results with physiological data are given.

KEY WORDS: Corrosion casting, scanning electron microscopy, urogenital system, blood vascular system, vertebrates.

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Introduction

The blood vascular system of the kidney is one main determinant of its function. Several studies cover the phylogenetical and ontogenetical aspects of the renal vasculature in different taxa of vertebrates (for reviews, see e.g., 3, 22, 44, 47, 48, 50, 52). Relatively few comparative or morphological studies exist on the functional adaptations of excretory systems of non-mammalians to their specific environments (6, 9, 15, 16, 17, 18, 35, 57).

Studies on renal blood vessels, like all studies on luminal organs, allow various approaches to the visualization of the investigated structures.

The methods most commonly used are: i) Sectioning methods for light- or transmission electron microscopy; ii) Freezing methods - freeze fracture/etching for scanning electron microscopy (SEM) or transmission electron microscopy or direct observation of frozen hydrated specimens on a cryostage, and iii) Injection methods, involving either clearing or corrosion of the surrounding tissue.

The optimal method to be used apparently depends on the specific question of interest. However, combinations of different methods greatly improve the reliability of morphological results and help in identification of artefacts.

In our studies on developmental and functional parameters of the renal vascularization in different vertebrate taxa, a combination of conventional histological paraffin sections for light microscopy, SEM of critical-point dried tissue and SEM of polyacrylate corrosion casts has been found most useful. From the great variety of casting media and procedures that are reported in the literature (for reviews see 20, 24, 31) a processing schedule, giving good results in all the vertebrates studied so far, had to be developed, as comparatively few studies on non-mammalians can be found in the literature (1, 2, 12, 13, 14, 15, 16, 30, 34, 37, 41, 43). Several problems concerning the comparability of the obtained results have therefore to be considered.

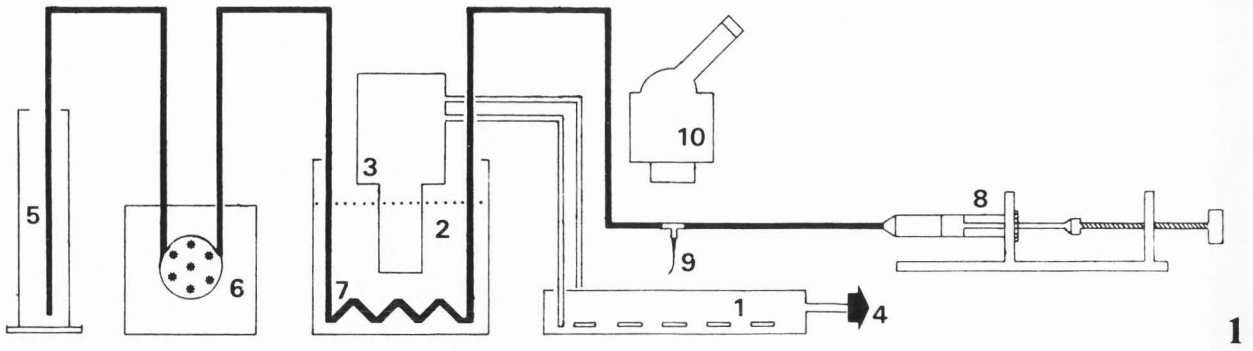


Fig. 1: Diagram of the setup for the preparations. The animal is placed on the operation desk (1) that can be heated from a water bath (2) with a thermostat-heater (3) by the heating line (white). The operation desk is drained (4). Perfusion is carried out from a graduated cylinder containing the Tyrode solution (5) by an adjustable peristaltic pump (6) and heated in a heat-exchanger (7) in the water bath. The syringe in its mechanical press containing the injection resin (8) is connected with a T-piece to the perfusion line near the catheter (9). Preparation is carried out with the help of a dissecting microscope (10).

Casting procedure

With the procedure as described here 14 different species, members of the classes of Chondrichthyes, Osteichthyes, Amphibia, Reptilia, Aves and Mammalia have successfully been investigated. Initial efforts on embryonic material are still in progress. The studied animals were male or female, adult, aroused and kept under laboratory conditions at least for a week. Their body weight ranged from 7.8g (lizard) to 1750g (mallard).

Anaesthesia was carried out in terrestrial animals by i.p. injection of pentobarbital (10mg/100g body weight), in aquatic animals by addition of ethyl-m-aminobenzoic acid (1g/10l - "MS 222"; Sandoz; Switzerland) to their respiratory water. The latter anaesthetic has to be applied with care as it may cause leaky gill vessels if applied too long. The dosages of both agents are usually lethal to the specimen, however, the time until respiratory depression and circulatory collapse occur is long enough for preparation of the heart and perfusion. The fast effect even in animals that are difficult to anaesthetize (e.g., turtles or Tench) favours such a high dosage.

Anticoagulants are used in low doses (0.1-0.2ml of heparin; 5000I.E./ml) only if the available material is limited or expensive to prevent thrombosis resulting from the preparation. In careful preparations, this step can be omitted in order to keep all parameters as normal as possible and to exclude potential side effects of heparin (46). No vasodilatators are used in order to approach the in vivo state of the vascular bed.

The animals are placed on a specially designed operation desk (Fig. 1) when anaesthesia is deep enough to start the preparation. The table is either heated (mammals, birds) to the specific body temperature of the animal or adjusted to keep the animal´s temperature at a constant physiologic value (usually a few degrees above room temperature) during the whole preparation (poikilotherms). In all cases the heart of the animal and the emergence of the aorta are dissected free, damaging as few vessels as possible. In fish this may seem disadvantageous as the gill system has to be passed by the casting resin. However, the fish kidney begins immediately caudal to the head and injecting via the dorsal aorta would therefore result in a partial loss of the structures of interest. Injecting via heart/aorta maintains the physiological blood route. From the great variety of cannulas that may be used, disposable polyethylene catheters, drawn to the appropriate diameter proved most suited as the danger of piercing the vessel can easily be avoided. This catheter is placed into the aorta (or aortic branch) via the left ventricle and secured there with a ligature. In reptiles, the right aortic branch is clamped.

Perfusion of the animal to flush the blood out of the vascular system with the appropriate Tyrode solution (10, 11, 19, 23, 49, 51) should fill the following demands:
1) isotherm; 2) isobar; 3) iso-osmotic, and 4) in accordance with the blood values for the main anorganic compounds and pH.

The volume of the Tyrode solution should be about three to five times the blood volume of the specimen (being estimated as 10% of the body weight). This quantity is sufficient to prevent thrombosis although the vascular system is usually not completely blood free. Oedemas or leaky vessels are prevented with this small amount of perfusate, the remainders of blood are pressed out by the casting resin. Perfusion is accomplished with a peristaltic pump that is calibrated according to the mean cardiac output rate of the animal (10, 11, 19, 23, 49, 51). The elasticity of the arterial walls facilitates perfusion as the pressure at the renal arteries is autoregulated, even if the perfusion pressure is slightly deviating from the acute value of the animal. Also the

pulsations of the peristaltic pump are partly compensated by these effects. The abdomen of the animal is left intact to approach natural intra-abdominal pressure relations during perfusion. Efflux of blood and Tyrode solution is allowed by cutting the posterior caval vein during onset of the perfusion.

After perfusion with the Tyrode solution, the casting resin is injected supravitally through the same catheter. Either methyl-methacrylate prepared according to Murakami (39), as modified by Lametschwandtner and Simonsberger (28), Mercox (Jap. Vilene Co.) or Mercox diluted with 20% methyl-methacrylate monomer (42) is injected by a syringe in a mechanical press (modified construction after Lametschwandtner et al., 29) until the outflowing casting resin at the cut posterior caval vein appears macroscopically free of blood/Tyrode droplets. Using the blue-coloured version of Mercox has the advantage that the filling of the liver can easily be observed, indicating completeness of renal filling. Mercox diluted is now routinely used by us as its low viscosity makes complete casts more easily obtainable, especially if the gill system of fish has to be passed by the resin before reaching the kidney. In all cases the injected amount has to be large enough to avoid trapping of liquid bubbles in the vessels or cylindrical casting of some vessel walls with a remaining lumen in the cast. The speed of the injection is controlled by observing the dilatation of the aorta during injection. This, however, is a matter of personal experience and some pressure monitoring device may be used if desired. Immediately after injecting the resin the posterior caval vein and the catheter are clamped and the whole specimen is cured in situ in a 60°C water bath overnight.

Corrosion of the specimen is carried out in daily changes of 10% KOH (55). The casts are rinsed in tap and distilled water and "ice embedded" at -20°C (24, 31). Selected areas of interest can easily be trimmed out of these ice blocks using a diamond cutting disc driven by a "mini tool" motor under stereomicroscopical control. Clean, defined cut surfaces can be produced in this way (Figs. 2,3).

The further steps in preparation after melting the ice at room temperature in a desiccator with silica gel blue and its drying (mounting, gold sputter coating, ...) are the same as conventionally applied to SEM specimens (see e.g., 12, 15).

A "JEOL-JSM 35 CF" scanning electron microscope that has been checked for spherical aberration and correctness of the magnification display, operated at 5-15kV, is used for the investigations.

Evaluation of Renal Corrosion Casts

Good casts can be judged by the presence of several criteria like: presence of impressions of endothelial nuclei, complete replication of peritubular portal capillaries (without blind endings), casting of glomerular mesangial matrix channels (being found in the gaps between glomerular capillaries - Fig. 4) and absence of round ended tips of blind ending vessels. Such casts can be investigated with quantitative methods. Interspecific comparisons of these quantitative morphological data may contribute to the understanding of the development of renal function. The inevitable methodological mistake in such measurements like polymerization shrinkage, dilatation of the vessels caused by the injection procedure, measuring inaccuracy due to the morphometrical method used, (e.g., 5, 31, 54, 55, 56) are in the same order of magnitude if the procedure is standardized as much as possible. Comparisons with quantitative results obtained by other methods (e.g., histology) are relatively limited as only freezing methods, when applied properly, can be assumed to represent in vivo size. Fixed and dehydrated kidney tissue shows shrinkage up to 60% of its initial volume, depending on the used procedure. Application of such additional methods, however, can provide useful, qualitative information for interpreting peculiarities of the casts (e.g., vascular sphincters, intra-arterial cushions, venous valves, etc.).

Several points have to be obeyed in quantitative studies using SEM to avoid methodological mistakes. Care must be given to parallel orientation of the feature to be measured with respect to the plane of focus of the microscope. Even a relatively simple parameter, like e.g., the largest glomerular diameter, may require stereophotogrammetry (Fig. 5) to quantify the error resulting from obliqueness of the measured distance (14, 15). The same demand applies to measurements of the cut surfaces of casts (Fig. 2) with planimetry or other stereologic methods (5, 15, 54).

A main advantage of SEM of microcorrosion casts is the wide range of magnifications that can be applied. With low power micrographs (Fig. 3) the organ level can be studied. The relationship of renal vessels to the aorta and vertebral column can easily be demonstrated, while this may be quite complicated in dissection or histology. Studies on the intrarenal capillary system using LM or TEM suffer from the high responsiveness of these structures to fixation (21). Quantitative studies on this important component of the renal functional unit (7, 8, 21, 27, 45) allow comparisons that may explain functional adaptation of basal excretory systems (15, 16, 17). Investigations on size and morphology of the glomeruli of different species show the wide range of differentiation of this structure in vertebrates. Amphibians were found to have the largest (Table 1) while the smallest renal corpuscles were found in birds (6, 14). Such differences cannot be explained by environmental adaptations alone. However, in closer related species, functional variation of the specific renal system can be observed. In turtles, e.g., a terrestrial species, shows fewer but larger glomeruli than a fresh water species in a given volume of kidney tissue (15, 16). The peritubular portal capillary system of the terrestrial species is

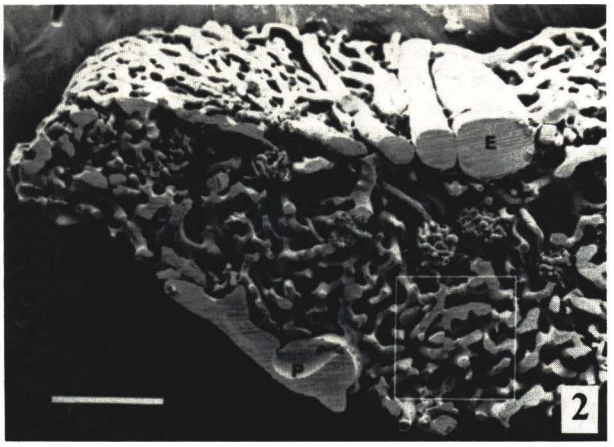


Fig. 2: Cranial view of a distal part of a section through a cast of the left kidney of a salamander. The afferent (portal - P) and the efferent (E) renal veins as well as several glomeruli can be seen. The area in the white square contains 29.7 ± 2.7 vol.% of peritubular capillaries. Bar = 500µm.

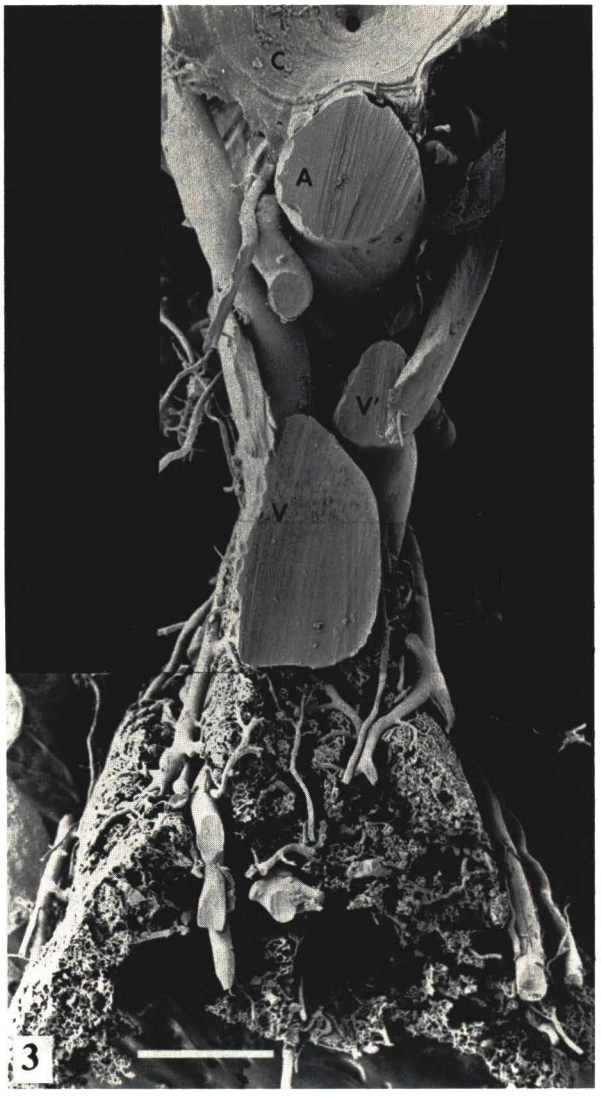


Fig. 3: Frontal section through the cast kidney of a Tench showing the topographical relationship of a vertebral corpus (C), dorsal aorta (A) and posterior cardinal veins (V, V´) with the kidney shortly posterior to the swimbladder. Bar = 1mm.

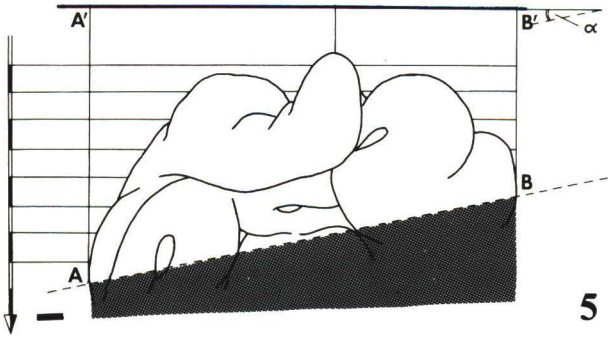


Fig. 5: Lateral projection of a stereophotogrammetrical "contour map" of a glomerulus. Note that the largest diameter (A - B) appears shortened by cos.α in the normal vertical projection of a single SEM micrograph (A´ - B´). Bar = 5µm; layer thickness = 5µm.

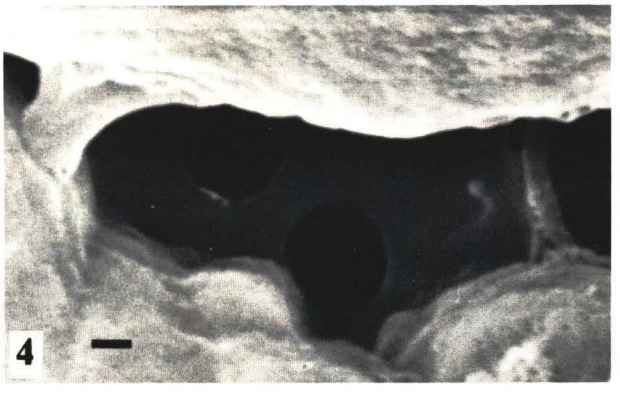


Fig. 4: Detail of a glomerulus showing casts of extravascular matrix lacunae (mesangial channels). Bar = 1µm.

Table 1: Mean glomerular diameters ± their standard deviation ($\bar{x} \pm$ s.d.) of 11 species of five different classes of vertebrates (n = number of measurements). Note the large differences between birds and amphibians. For ovoid glomeruli (e.g., birds) the shortest and longest diameter was measured.

Class: Species	n
Osteichthyes:	
Tinca tinca (L.)	34
Amphibia:	
Ambystoma mexicanum (Shaw)	26
Salamandra salamandra (L.)	27
Bufo bufo (L.)	26
Reptilia:	
Pseudemys scripta (Wied.)	121
Testudo hermanni (Gml.)	44
Iguana iguana (L.)	58
Podarcis peloponnesiaca (Bibr.& Bor.)	37
Aves:	
Anas platyrhynchos (L.)	42
Streptopelia roseogrisea (Sund.)	89
Mammalia:	
Rattus norvegicus (Berk.)	32

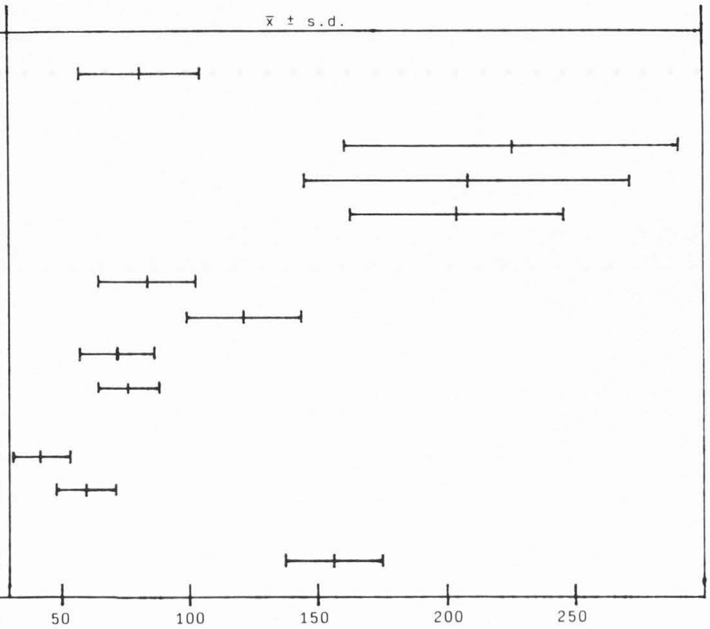
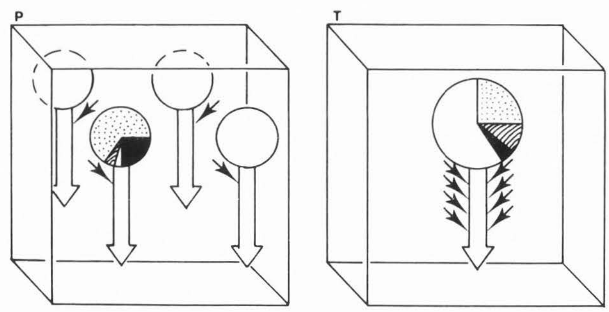


Fig. 6: Schematic comparison of equal volumes (cubes) of kidney of the Green Pond Slider turtle (Pseudemys scripta - P), living in fresh water and Hermann´s tortoise (Testudo hermanni - T) living in arid biotopes. Four glomeruli (circles) of a mean diameter of 83.1μm are found in P compared to one larger (mean diameter 121μm) in T. The peritubular network (arrows) is 2.5 times stronger in T. The nitrogenous excretion products are different in accord with the respective vascular development and environmental water supply (black - ammonia; dotted - urea; white - uric acid; hatched - rest).



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stronger developed (Fig. 6). These morphological results correlate well with physiological data on the excretory products of these two species (9, 38). The mesangial channel system (13, 15, 16, 26, 32) in the glomerulus (Fig. 4) consists of intercellular lacuneous gaps in the mesangial matrix. Though the function of this compartment is not yet fully cleared up, it has been found to form a connection between the capillary lumina, mesangial cells and macula densa of the renal tubule (4, 33, 36, 53).

General Considerations

A great variety of casting procedures is described in the literature. Macroscopical and light microscopical investigations on cast renal vessels date back to the 19th century (25). SEM of polyacryl casts was originally described for glomerular investigation (39, 40). This method is the subject of several modifications and is widely used in studies on luminal structures (see 20, 24, 31, 56). In comparative and quantitative studies on animals from different vertebrate taxa a few points should be considered in addition to the general criteria for corrosion casting (24). i) The whole preparation procedure should meet the in vivo situation of the specific animal as close as possible. This request may not be fully reached in some cases as not all data that are required may be reliable for less common species when taken from the literature. E.g., blood composition and systemic pressure may be the subject of large variations due to endogenic factors (e.g., hibernation, mating, stress, ...). Whenever such effects can be expected, these data should be checked before starting the preparations in a

representative sample. Furthermore, no additional parameters that are not essential for casting (vasodilatators, prefixation) should be introduced as the resulting effects may not be comparable in different species. The physiologic blood route should be followed by the casting medium. Injecting the whole vascular system from the aorta, as described here, follows this demand. However, in large animals it might be necessary to inject surgically exposed single kidneys. ii) The casting parameters should remain constant with respect to the technical aspects. The great variety of casting media and their differing technical properties (55, 56) impede comparisons of quantitative results of studies using different casting methods. All polyacryl resins mentioned above gave good results in our studies. However, the ease of preparation and its low viscosity favours Mercox diluted with 20% methylmethacrylate. Possible alteration of the surface of the cast by the macerating solutions can be minimized by application of 10% KOH no longer than absolutely necessary (55). Differences in the time required for corrosion resulting from different animal sizes can partly be avoided by removal of unnecessary parts after curing. Air drying of the specimen (in a desiccator with silica gel blue) never produced apparent distortion or fractures in our casts. Freeze drying or CPD of the specimen was therefore omitted. Observation of the cast in SEM should be carried out with acceleration voltages of less than 15kV as distortion or melting may occur with longer observations at high magnification if a too high acceleration voltage is applied.

Quantitative evaluations of corrosion casts from different animals can be made with a variety of stereologic techniques (5, 14, 15, 54) provided that the requirements mentioned above are regarded. The interpretation of such results has to be evaluated with care as apomorphic and plesiomorphic parameters contribute to the differentiation of vascular structures. Environmental and physiologic effects should always be taken into account. Additional usage of other morphological methods (dissections, histology, TEM, ...) does not only help in interpretation of a cast feature but also helps identifying artefacts and increases the evidence of the results.

Acknowledgements

This study was supported by FWF-Project P5364.

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Discussion with Reviewers

D.B. Jones: Did the authors notice size variation between outer cortical glomeruli and juxtamedullary glomeruli in the various species?
Authors: Significant size variations are found in birds (comp. Refs. 6, 14) between outer "reptilian-type" and inner "mammalian-type" renal corpuscles and obviously in the rat. However, although the other non-mammalians we studied sometimes showed large variations in glomerular size (e.g., Amphibia in Table 1) a structural differentiation into renal cortex and medulla is lacking and the different sized glomeruli are randomly distributed as far as we have seen. A probable tendency towards larger glomeruli being situated closer to the supporting vessel (terminal artery, intralobular artery) than the smaller ones remains to be verified by further investigations.

A. Lametschwandtner: How many specimens of each species did you cast and how many of them revealed good filling?
V.H. Gattone II: What number of animals were used for the generation of data in Table 1 and were these adult animals?
A. Lametschwandtner: You mention that 14 different species were casted, but in Table 1 you only refer to 11. Please comment upon the remaining species!
Authors: A minimum of four adult specimens, at least one of each sex have been used for the measurements represented in Table 1 (usually more - e.g., 18 specimens of Pseudemys scripta); only fully cast vascular systems were used. Depending on the species and on experience, usually about 20% of the preparations give unsatisfactory results (e.g., incomplete filling). For the species not included in Table 1 (Ameiva sp., Chelydra serpentina - reptiles; Acipenser ruthenus - chondrostean fish) we did not yet have enough specimens to be able to exclude individual variations, although the casts are of good quality.

D.B. Jones: I´m not sure whether the mesangial channels to which the authors refer are demonstrable channels which fill with injection mass or whether they represent potential channels which have been demonstrated experimentally to slowly transport macromolecules toward the glomerular hilus. Perhaps this could be explained.
Authors: We are currently studying these questions using colloidal gold in TEM sections. Preliminary results indicate that, at least in turtles and birds, the mesangium (that is more strongly developed than in mammals) contains a highly branched lacuneous system of matrix channels which may take up macromolecules, especially in response to disease, and can also be filled by casting resins. We hope that our further studies will provide more detailed information.

V.H. Gattone II: The investigators were very careful in their method of perfusion with physiological Tyrode solution in flushing the vasculature. However, since the vasculature was unfixed, was there any concern about the renal vascular effect of: a) anoxia during the operation and casting or b) toxic effect of the casting resin?
Authors: The approximate time span between perfusion (beginning anoxia) and starting of solidification of the casting resin is around 15 minutes. Vasomotoric effects during this cannot be excluded without usage of vasomotoric agents (e.g., vasodilatators) that may have no comparable effects in the different species. However, most of the lower vertebrates we use are relatively tolerant against short-time oxygen deprivation. Toxic effects of the casting media, disregarding long exposure effects such as possible cancerogenicity, hepatotoxicity, etc., are not reported in the literature. By appropriate admixture of catalyst, the time of contact between living vessel wall and still deformable casting resin can be restricted to a few minutes. Although the effects you mentioned may take place we think that their consequences on the appearance of the casts can be overlooked.

A. Kikuta: The wall structure of the blood vessels and those of their surrounding tissues may vary in each species. Were there any differences in dilation rate caused by resin injection among the species which the authors investigated? Have the authors compared the sizes of the glomeruli between those obtained by corrosion cast method and those by frozen method?
Authors: We currently have reliable data only on doves, where we compared freeze-fracture

replicas with corrosion casts. In this case both methods gave similar results within the limits of statistical standard error. We have not yet studied the other species.

A. Kikuta: What do the authors think of the effects of fixation prior to resin injection? Gattone and Evan (58) performed controlled perfusion fixation in such quantitative studies using corrosion cast methods and they stated that the controlled perfusion fixation is necessary for the quantitative data collection.
Authors: Working on a single species without changing the parameters of perfusion fixation, as the authors you mentioned did, will give comparable data as the inevitable fixation shrinkage can be assumed to be the same in all specimens. This may not be the case in comparative studies on different species. Furthermore, as no apparent benefit resulted from prefixation in our studies we omitted this step in order to keep the procedure as simple as possible.

Additional Reference

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