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<td>The impact of the architecture of the Dipodamys Merriami's inner medullas on the urine concentration and possible human applications.</td>
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THE IMPACT OF THE ARCHITECTURE OF THE Dipodomys merriami'S INNER MEDULLA'S ON URINE CONCENTRATION AND POSSIBLE HUMAN APPLICATIONS

By
Vinoo B Urity

A Thesis Submitted to The Honors College
In Partial Fulfillment of the Bachelors degree
With Honors in
Physiology
THE UNIVERSITY OF ARIZONA
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Approved by:
Dr. Thomas Pannabecker
Department of Physiology
Abstract:

Background: The cellular mechanism for the development of the osmotic gradient is not well understood in the inner medulla. With that said, this concentrating mechanism most certainly involves the interactions between the several different segments of each of the following: Loops of Henle, the collecting ducts, and the vasa recta. Mapping of the three-dimensional arrangement of all the inner medullary structures provides information as to how interactions, such as fluid and solute flows, between tubules and vessels lead to multiple countercurrent mechanisms that play critical roles in the concentrating mechanism.

Methods: Male Kangaroo rats were euthanized and their kidney’s dissected. The inner medulla was separated and imbedded. The human kidneys were acquired through organ donations. This tissue was sectioned, placed onto slides, and proteins labeled with immunohistochemistry. These slides were later imaged with an epifluorescent microscope and saved as .tiff images. These images were used as templates for image segmentation of renal structures to produce 3D reconstructions of the kangaroo rat kidney and human kidney inner medullas.

Findings: The kangaroo rats have a larger ratio of ATLs to DTLs, and a smaller pre-bend in comparison to Munich-Wistar rats (MW). The kangaroo rat pre-bend is shorter than the MW rat pre-bend (116 um vs.181um). A longer length of a kangaroo rat DTL is AQP1 positive (66% vs 36%). For both humans and rodents, tubular structures are arranged in a discrete pattern around the collecting ducts. In the rodents, the proximity
of tubular and vascular structures to the collecting ducts separates parts of the interstitium from the rest of the medulla.

**Conclusions:** Based on these data we can say there are two countercurrent systems that are in play within the rodent IM. One system relies on the nodal spaces while the other takes advantage of the intra-CD clusters. The humans lack nodal spaces and therefore are unable to use the first countercurrent system. More research into the 3D arrangement of structures is needed before an accurate human passive countercurrent system can be defined.
**Introduction:**

The urinary concentrating mechanism (UCM) is thought to be a countercurrent multiplication of an osmotic pressure difference sustained by active solute reabsorption from the thick ascending limbs (TALs) (1). However, active solute transport coupled with countercurrent flow does not explain the concentrating process in the inner medulla (IM), where the steepest osmotic gradient is generated as the ascending thin limb in the IM do not have active luminal transport of solute (2). Therefore, the generation of the IM osmotic gradient must involve countercurrent multiplication by thin limbs of Henle’s loops assisted by the countercurrent exchange by the vasa recta (6). However, no specific mechanism for the generation of the inner medullary osmotic gradient has gained general acceptance.

That said mathematical models developed by Dr. Layton show that a strong theoretical case can be made for a passive concentrating mechanism (4). Based on our prior Munich-Wistar rat data, we assume that the kangaroo rat will contain completely AQP1-negative, short-long DTLs and ATLs that should have essentially no osmotic water permeability; AQP1-expressing DTLs that have very high osmotic water permeability; DTLs that have essentially no NaCl permeability; ATLs and prebends that have high NaCl permeability; AQP1-expressing DTLs that have moderate urea permeability (5). Finding the exact placement of these structures (AVRs, DVRs, ATLs, DTLs and CDs) will help in determining how and which passive mechanism is in play.
As the architecture of the IM has not been well-defined for any species, and not at all defined for the *Dipodomys merriami*, the exact arrangement of the loops of Henle, vessels, and CDs are not completely understood. Our project bridges the gap between past comparative studies of renal architecture and theories of fluid and solute compartmentation, countercurrent systems, and the UCM. Understanding the three-dimensional positional arrangements of IM tubular and vascular structures, and the detailed organization of their transporters and channels will help complete the passive countercurrent hypothesis of the renal IM concentrating mechanism (5).
**Methods:**

**Animals:** *Dipodomys merriami* were trapped in the Arizona desert in the Santa Rita Range, approximately 40 miles south of Tucson with assistance from Dr. Eldon Braun. We trapped five mice; three of which were prepared for immunohistology.

**Kangaroo Rat Tissue Prep:** Animals were euthanized with CO₂ and their kidneys perfused through the aorta with phosphate buffered saline (PBS) and .01M periodate-lysine-paraformaldehyde (PLP) for 5 min. IM was dissected from the rest of the kidney, immersed in PLP fixative for 3 hr at 4° C, washed in PBS, dehydrated through an ethanol series and immersed in a solution of Spurr epoxy resin. After fixation, serial 1 μm transverse sections were cut starting from the OM-IM boundary to the papilla with every fifth section placed on microscope slides.

**Human Tissue Prep:** Two human kidneys were procured through organ donations. These kidneys were fixed using PFA fixative, dehydrated through a series of ethanol and xylene washes, and embedded into paraffin blocks. For one kidney, serial 5 μm transverse sections are cut after fixation, and placed onto microscope slides. The other kidney was cut longitudinally to see the overall distribution of proteins throughout the entire human kidney.

**Immunohistochemistry:** Immunohistochemistry was used to label orthologous proteins in *Dipodomys merriami*. We used polyclonal antibodies against the carboxyl terminal of human water channel aquaporin 1 (chicken AQP1) to act as a marker for DTLs, while antibodies against the human water channel aquaporin 2 (goat AQP2) labeled CD. Antibodies against rat kidney-specific chloride channel (rabbit CIC-K) binds to ATLs
(ClC-K1) and CDs (ClC-K2 in the IM). FITC autoflourescence, which shows all tubules, will be utilized to find the AQP1 null DTLs by showing all tubules not marked by the aforementioned antibodies (5).

To label the blood vessels of the kidney, we used affinity-purified polyclonal antibodies raised in rabbits against the COOH-terminal region of the urea transporters UT-A and UT-B. We used the antibody against UT-A to label DTLs near the OM-IM boundary and the UT-B antibody was used to label DVR all throughout the kidney. AVR were identified in a two step method. FITC-conjugated lectin (wheat germ lectin) labeled both the DVR and AVR and since we can identify DVR with the UT-B antibody, and all other loop segments by way of additional antibodies, we were able to differentiate the AVR. Also, as the AVR are fenestrated vessels, they can easily be identified using electron microscopy.

We are confident these antibodies labeled orthologous proteins in *Dipodomys merriami* since mammalian urea transporters, aquaporins, and ClC chloride channels are highly conserved proteins (3). Also, the specificity of antibody labeling was confirmed by comparing *Dipodomys merriami* proteins with the orthologs using western blotting.

In the human tissue, antibodies against the carboxyl terminal of human water channel aquaporin 1 (chicken AQP1) acted as a marker for DTLs, the human water channel aquaporin 2 (goat AQP2) antibodies labeled CD, the human urea transporter UT-B antibodies marked the DVR, and the chloride channel subunit barttin (ms barttin)
labeled DTLs/ATLs/CDs. We were able to identify the ATL as it was the only tubule marked by barttin and not marked by the aforementioned antibodies.

**Three Dimensional Reconstruction:** We first created two sets of montage images. One set was obtained by capturing each of three wavelengths from each tissue section, depicting expression of AQP1, AQP2, and CLC-K while the other was obtained by capturing each of three wavelengths from each tissue section, depicting expression of AQP2, UT-B, and lectin labels. We used an Olympus IX70 epifluorescence microscope, a 10X objective, and a motorized stage for the image capture. Serial image alignments, tubule segmentation, and three-dimensional reconstructions were produced with Amira visualization and volume modeling software.

Three separate stacks of digitized, serial, two-dimensional tiff images were generated by capturing AQP1, AQP2, and CLC-K1 immunofluorescence from the first set of serial tissue sections, and three additional stacks of images were generated by capturing AQP2, UT-B, and lectin immunofluorescence from the second set of serial tissue sections. This process yields a total of six stacks of images that enable us to outline the entire length of all DTLs, ATLs, CDs, DVR, and AVR. Tubule segmentation, a tool in the Amira reconstruction software, was used to produce a binary image by assigning all pixels of an individual tubule to the foreground and provide a continuous surface representation of each tubule.

This process was conducted with 600 slices of the kangaroo rat tissue giving a detailed reconstruction of roughly 3mm of its inner medulla. Reconstructions were not conducted with the human tissue.
**Results:**

**Basic Architecture of Kangaroo Rat:** From the outer zone of the IM in the kangaroo rat, CDs are arranged in cluster ranging from three to six CDs per cluster (Fig 1). As we continue further down the medulla towards the papilla, the CDs within the clusters begin to merge and eventually the clusters themselves coalesce to form the papillary ducts of Bellini, where the final urine exits the kidney. Within each cluster, the Cl-CK1 positive ATLs are arranged uniformly around individual CDs (Fig 1). The AQP1 positive DTLs are scattered throughout the periphery of these clusters. While AQP1 is also seen in DVRs, they also express UT-B and their relationship with the cluster needs further research. As we descend further into the medulla, DTLs descend along the corticopapillary axis outside the CD clusters, whereas their ascending segments ascend either outside or inside the CD clusters. From the electronmicrographs, we see AVR, as identified by their fenestrations, also are arranged uniformly around individual CDs inside each CD cluster. The close proximity of the ATLs and the AVRs to individual CDs partitions part of the interstitium surrounding the CD from the rest of the medulla laterally. These isolated pockets have been termed interstitial nodal spaces (Fig 2)

**Loops of Henle Ratios:** A loop of henle consists of a DTL (AQP1+) descending towards the papilla, reaching a pre-bend and forming a hair pin loop to ascend back towards the cortex as an ATL (ClCk+). At each transverse level of the inner medulla the number of ClC-K positive tubules is greater than the number of AQP1 positive tubules. Comparatively, the ratio of ATLs:DTLs is much higher in the kangaroo rat than in the Munich Wistar, or the common lab rat (5). In addition, the ATLs in the kangaroo rats do not reach as far into the papilla as they do in the MW rat.
**Pre Bend Lengths:** In the kangaroo rat, CIC-K is expressed in the pre-bend segment (the terminal end of the DTL) along a length of $116.5 \pm 5.1 \, \mu m$ (mean ± SE). This length is a fixed length for all loops of Henle, regardless of the axial depth at which they form their bend. Comparatively, the MW rats’ pre-bend measures $180.9 \pm 5.7 \, \mu m$ (mean ± SE).

**DTL Lengths:** The DTLs express AQP1 only if they penetrate deeper than 500 microns, on average, into the inner medulla. These segments stop expressing AQP1 as they approach their pre-bend. The length of the AQP1 positive segment is tied to the length of the overall segment from the OM-IM boundary to the pre-bend segment (Fig 3). The mean AQP1-positive fractional length for loops between 2365 and 2615 microns long was $0.66 \pm 0.01$ (mean ± SE) with a range from 0.64 to 0.71. This is quite different from the MW rat which has an AQP1-positive fractional length of $0.36 \pm 0.01$.

**Preliminary Human Results:** The overall architectural motif of the arrangement of ATLs around individual CDs and DTLs around CD clusters is conserved between the rodents and humans (Fig 4). The CIC-K1 positive ATLs are arranged uniformly around individual CD while the AQP1 positive DTLs are scattered throughout the periphery of each cluster. The ATLs and AVRs are not as closely associated with individual CDs as they are in the rodent kidney. They do not section off part of the interstitium surrounding the CD from the rest of the medulla. As a result, there are no nodal spaces in the human medulla.
**Discussion:**

Our project examined the *Dipodomys merriami* renal inner medulla using immunohistochemistry, immunoblotting (thanks to Juan Siordia of the Pannabecker Lab), and software reconstruction to better understand the effects of the placement of renal architecture on urine concentration. Antibodies against segment-specific proteins indirectly indentify the tubule or blood vessel. Antibodies against aquaporin 1 reveal AQP-1-positive descending thin limbs, ClC-K1 chloride channel identifies ascending thin limbs, aquaporin 2 shows collecting ducts, urea transporter B reveals descending vasa recta and FITC conjugated wheat germ lectin recognizes the ascending vasa recta.

Based on the segments indentified by the Immunohistology, we propose two anatomically distinct countercurrent systems (Fig 5). Countercurrent system one works within CD clusters and relies on the presence of nodal spaces (INS). CDs reabsorb urea and ATLs reabsorb NaCl and urea from the interstitium. Water follows the solute. As the area surrounding CDs is isolated from the rest of the IM interstitium to some degree, a very high concentration of NaCl and urea is thought to be formed within the INS. Once the concentration of solute in the nodal space becomes greater than the concentration of solute in the nearby AVRs, the NaCl, urea and water enter the AVR. This takes the water and its solutes towards the OM-IM boundary where the concentration in the interstitum is low. At this point, the solutes are recycled back into the CDs and DTLs to start the cycle once again. Countercurrent system two uses the same identical principle except that it works outside each CD cluster. When the ATLs reabsorb NaCl and urea on the side not adjacent to the CD cluster, this solute gets carried laterally through the kidney. This diffuse solute then encounters an AVR. The AVR takes it to the OM-IM
boundary where it’s recycled back to the DTLs. The main difference between the two systems is that the uptake into the AVR is not quite as fast in countercurrent two as the solute is not sequestered near the AVR.

The comparative studies show a longer length of AQP1 positive DTL segment in kangaroo rats. This suggests that in that animal there is a larger area over which water can be exchanged between the DTL and the interstitum. As a kangaroo rat produces a higher urine concentration than a MW rat, this larger AQP1 segment might be critical to the development of a higher concentration gradient along the corticopapillary axis. Theoretically, if the overall flow of water per unit of the DTL is the same in both rodents, then the large AQP1 segment means that kangaroo rat is able to remove more water thereby making the NaCl in the DTL far more concentrated. This higher NaCl concentration within the DTL will cause a higher NaCl flux at the pre-bend segment due to the higher osmotic gradient.

While the kangaroo rat AQP1 segments are fractionally larger, they have a shorter absolute prebend length than the MW rat. As modeling studies predict that transepithelial chemical driving forces result in the most robust NaCl reabsorption along the prebend and postbend equivalent length portion of the ATL, the area over which the NaCl will be able to be exchanged is far smaller in the kangaroo rat in comparison to the MW rat (4). This implies that the shorter prebend has some impact on the overall osmotic gradient, possibly reabsorbing a higher fractional NaCl load along a narrower corticopapillary axial length compared to the MW rat.
The importance in understanding if the rodent countercurrent systems can be applied to humans is self evident. All drug testing is initially conducted on mice and rats to test its effectiveness. A key aspect that affects a drug’s efficacy is how it’s processed and as the biological effect of organic cations such as the active compounds in drugs may be dependent on the osmotic gradient, it’s imperative to compare and contrast the rodent kidney to the human kidney. The fact that the human kidney does not possess nodal spaces means that the countercurrent systems that define osmotic gradients in rodents cannot be directly applied to humans. Further research is needed to find a mathematical model that can be applied to humans so that any information derived from rodent studies can easily be translated into a human model.
References:


Figure 1: Transverse section of the central core region of the inner medulla near the OM-IM boundary of Dipodomys merriami. CD (AQP2/blue), AQP1-positive DTL (AQP1/red), and ATL (CIC-K1/green). Nine CD clusters are predicted from preliminary three-dimensional reconstruction (each cluster is outlined by white borderlines).
Figure 2: Electron micrograph showing transverse section of CD, AVR (asterisk), and ATLs from Dipodomys merriami IM. Five AVR abut central CD. Interstitial nodal spaces (X) are formed between CD, AVR and ATLs. Boxed area is enlarged in left panel to show fenestrae (arrow) that exist in the four AVR shown. Scale bars; right 10 μm, left 500 nm.
Figure 3: AQP1-positive fractional length of inner medullary DTL segments for Dipodomys and Munich-Wistar rat. The AQP1-positive length is variably proportional to the length of the inner medullary thin limb segment, measured from the OM-IM boundary to the bend of the loop. Line fit to exponential.
Figure 4: Transverse section of the inner medulla from the bottom third of the human kidney. CD (AQP2/large yellow), AQP1-positive DTL (AQP1/red), DVR (UT-B2/small yellow), ATL and AVR (wheat germ lectin/white). The overall architectural motif is similar to that seen in Fig. 1.
Figure 5: *Three-dimensional reconstruction suggested anatomically separate urine concentration countercurrent system*

**Countercurrent System 1 (Rodent Only)**
Location: OZ1 and OZ2 - Within CD clusters

Components:
1. Collecting Ducts  
   -axial descending flows
2. Intracluster Ascending Vasa Recta  
   -axial ascending flows
3. Intracluster Prebend/Postbend ATL  
   -local and transient NaCl delivery at bends

Function and Attributes:
1. Raise osmolality of CD tubular fluid by confining NaCl, urea, and water within CD clusters.
2. AVR are low-resistance sinks for CD absorbate that is carried to higher axial levels.
3. High-osmolality AVR fluid rises to progressively higher axial levels where it promotes fluid reabsorption from interstitial nodal spaces.
4. Diffusion of urea from AVR at progressively higher axial levels may limit diffusion of urea from CDs, leading to increased delivery of urea to deeper axial levels.

**Countercurrent System 2 (Rodent and perhaps Human)**
Location: OZ1 and OZ2 - Outside CD clusters

Components:
1. Intercluster Descending Thin Limbs
2. Intercluster Descending Vasa Recta
3. Intercluster Ascending Thin Limbs
4. Intercluster Ascending Vasa Recta
5. Intercluster Prebend/Postbend ATL

Function and Attributes:
1. Remove water from AQP1-positive DTLs and concentrate NaCl in DTL for reabsorption by prebend and ATL postbend in the interstitial nodal spaces.
2. Efflux of NaCl and urea from ATL and AVR promote water reabsorption from DTL.
3. AVR are low-resistance sinks for absorbate that is carried to higher axial levels in IM and OM.
4. NaCl and urea reabsorption from intercluster ATLs (CCS 2) may face lower interstitial NaCl and urea concentrations than intracluster ATLs of CCS 1, thereby favoring NaCl and urea reabsorption within intercluster region.

*DVR and AVR may not be representative of vasculature engaged in classical countercurrent exchange.*
Table 1: Comparative Measurements: Kangaroo Rat vs. Munich Wistar Rat

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<th>Kangaroo Rat</th>
<th>Munich Wistar Rat</th>
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<td>DTL (um)</td>
<td>2403</td>
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<td>Water Permeable Segment (um)</td>
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