Drug Treatment of Kidney clip-induced hypertension in rats; Peroxidase tracer spot frequency in rat aorta after chemically induced apoptosis

Simon Kleinbart
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Drug treatment of kidney clip-induced hypertension in rats.

Peroxidase tracer spot frequency in rat aorta after chemically induced apoptosis.

Thesis
Submitted in partial fulfillment of
the requirement for the degree

Master of Engineering (Chemical)
at
The City College of New York
of the
City University of New York
by

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August 2011
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Drug treatment of kidney clip-induced hypertension in rats.
Peroxidase tracer spot frequency in rat aorta after chemically induced apoptosis.
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Table 1.1 Coefficients of variation for treated and untreated rats
**Abstract**

Kidney clip-induced hypertensive rats were treated with two classes of anti-hypertensive drugs: beta-blockers and calcium channel blockers. Their blood pressures were monitored for several months, as were those of normotensive control rats. The hypertensive rats responded to treatment with drops in blood pressure, in some cases of greater than 70 mmHg. The variability of blood pressure was found to be similar in all three groups of rats.

Horseradish peroxidase spot size experiments were performed on rats that had been treated with cytotoxic agents to induce apoptosis. Spot frequency was observed to be lower in these treated animals than in control subjects. We propose explanations for this unexpected result.
Chapter 1: The effect of beta blocking and calcium channel blocking anti-hypertensive drugs on kidney clip-induced hypertensive rats

1.1: Introduction

Another student in the lab, Dr. Jimmy Toussaint, had investigated the role of aquaporin-1 (AQP1) in the progress of atherosclerosis, a topic addressed in great detail in (26). One aspect of that work was the effect of hypertension on the expression of AQP1 in rat endothelial cells; it increased such expression. As one of several controls, he sought to induce hypertension in eight rats, reverse the hypertension with drugs, and then measure aquaporin-1 expression. To this end, he performed the two clip, one kidney Goldblatt procedure on eight male Sprague Dawley rats. The purpose of this control experiment was to rule out the possibility that some aspect of this surgery aside from the elevated blood pressure it induces had an independent effect on AQP1 expression. All animal work was approved by the Institutional Animal Care and Use Committee (IACUC) of City College. In this procedure, described in Frei (6) and Mohring (15) the abdominal cavity of the rat is opened and a silver clip with a gap width of 200 microns is placed on the renal artery. This restricts blood flow to the kidney and induces various hormonal changes that eventually result in hypertension. Six of the animals developed hypertension, and it was my goal to medicate them to lower their blood pressure.

Toussaint had already performed a version of this experiment in which the drug of choice was captopril, an angiotensin converting enzyme (ACE) inhibitor. In that study, hypertensive rats that had been restored to normotensive with captopril were found to have far lower levels of AQP1 than both healthy, normotensive, untreated rats and sham-operated rats (in the sham operation,
every step of the Goldblatt procedure is performed except for placement of the arterial clip).

Toussaint therefore concluded that the Captopril had an independent effect on AQP1. He later conducted a literature review and found published evidence (31) of another ACE inhibitor’s negative effect on AQP1 expression (26).

1.2: Materials and Methods

In light of these considerations, drugs of different classes were selected for this batch of six hypertensive rats. The batch was subdivided into two groups, each including one rat with blood pressure above 200 mmHg and two with blood pressure of approximately 160 mmHg. One group was treated with generic atenolol (Teva), a beta blocker, and the other with diltiazem (Teva or Mylan), a calcium channel blocker.

The goal, then, was to lower these rats’ blood pressures. Three were given generic atenolol, a beta blocker in a dose range of 10 to 140 mg/kg/day. The other three were given generic diltiazem, a calcium channel blocker in a dose range of 40 to 200 mg/kg/day. The doses were increased gradually, and all rats were monitored closely by researchers and animal facility staff for signs of acute illness or distress. The drugs were purchased from local retail pharmacies in pill form and crushed and ground into powder in a mortar and pestle. Initially, both drugs were added to the rats’ drinking water. To achieve the intended dose, the rats’ daily water intake was measured over several days and the concentrations adjusted accordingly. Unfortunately, as noted in (6), rats have been known to avoid aqueous diltiazem solution, even at the risk of dehydration. Indeed, such was the case in our study, leading to two problems: the rats became dehydrated, and they did not receive the intended dose of diltiazem. Additionally, at high enough doses, even the atenolol solution was avoided, leading to some dehydration in that group as well. At various
points in the study, dehydrated rats were given Pedialyte (Abbot Labs), a sweetened cherry flavored beverage fortified with electrolytes, as a short-term antidote to dehydration.

To combat the less acute but more fundamental problem of how to have the rats ingest the drug, we decided to mix the diltiazem into a treat for the rats. On the advice of the institutional veterinarian, we initially selected peanut butter (Associated brand, purchased at a local supermarket) as this treat. However, this proved ineffective, i.e. the rats ate either some or none of the peanut butter and could not be assumed to be receiving the intended dose. Next we tried replacing the peanut butter with Bio-serv bacon flavored Transgenic Dough Diet, a calorie dense diet supplement or replacement with the texture of cookie dough that is designed to stimulate appetite. This proved much more popular with the rats, meaning they almost always finished the entire portion, and had the added benefit of countering weight loss. See (15) for other evidence of lower body weight in hypertensive rats. Eventually, all rats in the calcium channel blocker group and one in the beta blocker group received this Transgenic Dough Diet.

Another problem encountered was partial response. That is, blood pressure decreased in all animals treated with the initial dose but did not reach normotensive levels. The first step taken to address this problem was to simply increase the dose, and for the beta blocker group rats this eventually proved sufficient. However, in the calcium channel blocker group, blood pressure remained above normotensive levels even at higher doses, and so a second drug in the same class, amlodipine (Camber Pharmaceuticals), was added to those rats’ diets in a dose ranging from 1 to 10 mg/kg/day. Again, the dose was increased gradually, and all animals were closely monitored for signs of acute illness or distress.
Systolic blood pressure was measured by a non-invasive inflatable tail cuff method (ADInstruments). We did not monitor diastolic blood pressure, because these readings are less accurate and less reproducible by tail cuff measurements.

**1.3: Results and Discussion**

The graphs below represent systolic blood pressure recordings taken before the procedures and approximately once a week between the surgery and Toussaint’s AQPI experiment, which involved euthanizing the rats. The measurements were taken by Toussaint and Chirag Raval. In all the graphs, the first measurement was taken before the surgery. The surgeries were performed between September 4 and September 11. The six hypertensive rats were first given medication in late October and their doses were gradually increased over subsequent weeks and months. The values reported are averages of 4 to 15 measurements taken in one sitting, with error bars representing positive and negative spread about the mean. For the control rats (figs. 1.7-8), after 6 consecutive sets of post-surgical measurements all showed normal blood pressure, no more measurements were recorded until just prior to euthanasia in the next stage of the experiment. There is also a month-long gap between the last two measurements in figure 1.6. This represents a time when that particular rat showed various signs of illness, including low body weight and frequent dehydration, and its pulse and blood pressure could not be detected by our measuring equipment.
Figure 1.1: Blood pressure time course of rat A

Surgery: 9/11

Initiation of drug treatment: 10/19
Figure 1.2: Blood pressure time course of rat B

Surgery: 9/11

Initiation of drug treatment: 10/19
Figure 1.3: Blood pressure time course of Rat C

Surgery: 9/12

Initiation of drug treatment: 10/19
Figure 1.4: Blood pressure time course of Rat D

Surgery: 9/12

Initiation of drug treatment: 10/19
Figure 1.5: Blood pressure time course of Rat E

Surgery: 9/10

Initiation of drug treatment: 10/19
Figure 1.6: Blood pressure time course of Rat H

Surgery: 9/12

Initiation of drug treatment: 10/19
Figure 1.7: Blood pressure time course of Rat F

Surgery: 9/4

No drug treatment
Figure 1.8: Blood pressure time course of Rat G

Surgery: 9/10

No drug treatment
First note the different baseline blood pressure levels recorded about one month after surgery, before initiation of drug treatment. The two controls in this study underwent the full Goldblatt procedure, including placement of the clip, but failed to develop hypertension. Additionally, among the hypertensive animals, two were severely hypertensive (systolic blood pressure (SBP) > 200 mmHg) and four only moderately (SBP ~ 160 mmHg). Slight errors (on the order of tens of microns) in the gap spacing of the clips, as well as small variations in renal artery diameter among the animals, can go a long way toward explaining this variation, which has been found by other investigators (6, 9). Frei, for example, reports that only 60% of his kidney-clipped rats developed hypertension, and among the hypertensives there was a 12 to 14 point spread about the mean systolic blood pressure (6). It is reasonable to conjecture that, as the blood pressure increase is induced by constriction of the renal artery, variations in the gap width of the clip and the diameter of the vessel, which lead to variations in the level of constriction, would in turn cause variations in the level of hypertension. Also consider rat weight at the time of surgery, at least the most intuitively obvious indicator of vessel diameter. The graph below plots ultimate blood pressure as a function of rat weight at surgery. While there is a good deal of scatter and the total number of subjects is a rather low eight, the graph is at least suggestive of a correlation. Unfortunately, information on the deviation of the actual gap width of the clips from the attempted 200 microns is unavailable. It could be instructive to plot that data against ultimate
levels of hypertension and see whether it is a clearer predictor than rat weight.

![Mean systolic blood pressure 5 weeks after surgery (mmHg)](image)

**Figure 1.9: blood pressure versus rat weight at surgery**

Next, note the initial drop in blood pressure shortly after initiation of drug treatment at the end of October in all six cases. These drops, in all cases except case C, were not all the way down to normotensive levels. Also, in the diltiazem group, these decreases were followed shortly by relapses, as more diltiazem was added to the rats’ drinking water (in attempt to lower blood pressure still further, to normotensive levels) and they stopped drinking.

Some results seem almost obvious upon a close reading of the literature, others are less readily apparent but help to explain how previous experiments have been designed, and a few are truly novel. First, all rats responded to drug treatment at typical doses with decreases in blood pressure
in good agreement with previously reported results. Next, rats do tend to avoid diltiazem, as well as atenolol at high enough doses, even at the risk of dehydration. It should be pointed out, however, that this study was not the first to attempt to administer diltiazem via drinking water.

This study, as mentioned above, used pill forms of the drugs purchased from a local pharmacy, mainly for reasons of financial expense. These pills contain, by weight, far more inactive material than active (by a factor of approximately 2 to 40, depending on the drug). First there is the remote chance that so-called inactive ingredients actually have some effect that may introduce artifact. More troubling, however, is the tendency of the starches and other components to precipitate out of solution. One hopes that the precipitate contains only fillers, coloring and such and that all of the medically active substance remains dissolved in solution, but this is difficult to verify and, indeed, was not verified for this study. Another decision made for reasons of cost was to deliver the drugs via drinking water, as injecting the solution directly into the animals’ mouths would have required either recruiting trained animal handlers or investing in training the researchers involved in the study. In addition to the dehydration problem mentioned above, the rats grew over the course of the study and began to drink more than earlier measurements indicated. Further, variations in severity of the rats’ renal hypertension, a disease known to cause thirst (15), also led to changes in water intake. These factors help explain why other groups went to all the trouble and expense of obtaining pure forms of the drug from chemical suppliers and administering them to their animals by the more standardized method of daily oral injection. These concerns must certainly be taken into account in planning future work.

One finding bears mentioning, because it was not observed in a review of the literature on treatment of hypertension in rats with these drugs. This study found that, at high enough doses (which obviously did not prove lethal, or even immediately harmful), drops in blood pressure of
more than 70 points were possible with both atenolol and the diltiazem/amlodipine combination. One rat was even restored all the way from severely hypertensive to normotensive, although that piece of data should be treated with great care, as that rat exhibited other signs of illness for most of the last two months of the study, and its blood pressure proved unusually difficult to measure.

1.4: A note on blood pressure variability

After completing the above blood pressure study, our group learned, in private communication with pharmaceutical industry researchers, of interest in not only the level of blood pressure but also the level of variation of blood pressure in drug treated hypertensive subjects. This is in addition to the well-known problem of error in indirect blood pressure measurement. Since they have already studied spontaneously hypertensive rats, they are especially interested in kidney-clipped hypertensive rats, as these are not genetic mutants and are, therefore, considered more representative of the general rat population. Millar-Craig (14), referring to human patients, noted this issue in 1978, and our experience in this study confirmed that the problem has not gone away since. Beyond this, there is a tendency of blood pressure to vary as function of different factors, such as season (2) and time of day (12). In this study, since measuring variability was not the original goal, no precautions were taken to ensure that measurements were consistently taken at the same time of day. Depending on the researchers’ schedules and how long it took for the rats to become sufficiently calm and sedentary to allow for measurement, the recordings were taken in the morning, afternoon, or evening.

Obviously, some understanding of how blood pressure fluctuates is necessary for effective monitoring and treatment of hypertension. In addition to that, however, increased variation in blood pressure is a known indicator of hypovolemia (22), and decreased variation may be
important as a predictor of kidney disease (5), indicating that this measure may be an important
independent health factor. Clearly, then, if the goal of drug therapy of hypertension is to return
patients to the level of health and functioning of normotensive patients, then comparing blood
pressure variation in untreated normotensives and treated hypertensives is a reasonable next step
after bringing the mean pressures of the two groups within the same range.

To this end, we retrospectively examined the variability of blood pressure levels in our three
groups of rats: beta blocker treated (n=3), calcium channel blocker treated (n=3) and
normotensive control (n=2). Following Balansard (1) and others, we calculated coefficients of
variability (CV) for the eight series of measurements. This measure is simply the standard
deivation divided by the mean. Included were all data for the normotensive rats and data
collected after approximately two weeks of drug treatment for the hypertensives. The results are
tabulated below.
Table 1.1 Coefficients of variation for treated and untreated rats

<table>
<thead>
<tr>
<th>Beta Blocker</th>
<th>Calcium Channel Blocker</th>
<th>Normotensive Control</th>
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<tr>
<td>Rat A, Figure 1.1</td>
<td>0.0558</td>
<td>Rat D, Figure 1.4</td>
</tr>
<tr>
<td>Rat B, Figure 1.2</td>
<td>0.163</td>
<td>Rat E, Figure 1.5</td>
</tr>
<tr>
<td>Rat C, Figure 1.3</td>
<td>0.0580</td>
<td>Rat H, Figure 1.6</td>
</tr>
<tr>
<td>Rat F, Figure 1.7</td>
<td>0.133</td>
<td>Rat G, Figure 1.8</td>
</tr>
</tbody>
</table>

A cursory look at the above table brings a single word to mind: noise. The two normotensives have widely divergent CV’s, and four of six of the hypertensive CV’s fall within or just outside (0.133 versus 0.136) that range. One reassuring entry is the 0.190 for one of the calcium channel blocker treated rats. This rat’s high variability corresponds to the observations and experience of all researchers involved in the project. It exhibited dehydration, low body weight, and other signs of ill health for much of the study; its blood pressure proved far more difficult to measure than those of the other rats; and a plot of its blood pressure measurements over time (figure 1.6, rat H) looks almost sinusoidal.

Also reassuring is a look at the literature on human blood pressure measurement. Peixoto (21) took indirect blood pressure measurements of hemodialysis patients and included those currently taking antihypertensive drugs as long as their drug regimens remained unchanged for the duration of the study. He found CV’s based on their systolic blood pressure ranging from 0.075 all the way to 0.167, depending on the measuring procedure. Mancia (13) excluded all patients
with major diseases aside from hypertension, excluded those who had been treated with antihypertensive drugs, measured blood pressure by a direct invasive technique, and used a study population of 89 people. These precautions yielded long term systolic blood pressure variability, as measured by CV, of 0.08 to 0.10. (As a point of interest, the lower variability was seen in the severely hypertensive group.) Musso (16) notes that office sphygmomanometry is subject to “error as high as 38 mmHg.” And remember that rats, even in a warm, dark and quiet environment, cannot be expected to hold still, although we did attempt to encourage this by providing the rats with a quiet, warm, and dark environment for approximately an hour before and during the measurement routine.

In light of these considerations, the values in the table above, while they unfortunately fail to say anything definitive about the differences in blood pressure variability in normotensive and treated hypertensive rats due to the small sample size, do suggest that the methods selected for monitoring the rats’ blood pressures yielded results well within reported ranges.

One methodological point does bear mentioning. The coefficient of variability is essentially a normalized standard deviation. As such, it indicates of how far observations vary from the overall mean. It seems a perfectly appropriate (if somewhat blunt) measure for the medical studies cited above: the blood pressure is not expected to change, and this value quantifies the deviation from that expectation. Similarly, one can imagine a study similar to ours in which rats are given a constant dose of an antihypertensive drug, except, perhaps, for adjustments to account for weight changes. There too, we would expect a constant mean with some variation, and we could measure this variation. However, in our case, the rats’ doses were regularly increased until they became normotensive, or as close as we considered possible, at which point they were euthanized in the next stage of the experiment, rather than being maintained at that
dose and monitored for an extended period of time. There were also points at which the rats’
doses effectively decreased, for various reasons described above, e.g. they stopped eating or
drinking the drug-containing substance. If one were to partition the data into different regions
based on dose, regions in which one would expect a constant mean, one would in most cases find
only one or two sets of measurements between dose changes, for the following reasons. 1)
Measurements were taken once or twice a week, while dose changes often took a number of days
to implement due to rat taste preferences. 2) The experimental protocol called for rat euthanasia
to be carried out approximately one week after a stable lower blood pressure had been attained,
before the rats reached a critical age and weight. This did not allow us the time necessary to
monitor the rats’ blood pressures during extended drug treatment at a constant dose.

In light of this, if the treated rats had shown greater variability than the normotensives, it would
be unwarranted to ascribe this increase to the drugs. However, the treated rats, with the exception
of one with other health problems, showed variability quite similar to that of the normotensives
(slightly smaller for the beta blocker group and slightly larger for the healthy rats in the calcium
channel blocker group). This suggests two ideas. First, CV may be a fairly crude measure of
variability. Second, and more interestingly, the variability introduced by an antihypertensive
drug regimen in a small sample set seems to be only slightly greater than that of the ordinary
dynamic nature of physiology. One also needs to assess the effect of measurement error, but
recall Mancia’s CV’s of 0.08 to 0.1 even when measuring blood pressure directly.

The portion of the experiment that seeks to quantify AQP-1 expression in these rats’ aortic
endothelial cells is currently underway, under Toussaint’s direction.
1.5: Conclusion

If we were to set out to quantify blood pressure variability, we would design the study differently. We would use the same method of hypertension induction and probably the same drugs to treat it. However, we would be careful to consistently measure blood pressure at the same time of day. Even more importantly, we would decide on a dose, tune it according to rat response, and then maintain the rats at that dose for a number of weeks or months, during which time we would continually monitor blood pressure.
Chapter 2: HRP spot frequency in rat aorta after chemically induced apoptosis

Introduction

For decades now, researchers such as Chuang (4), Stemerman (25), and Shou (24) have used tracer studies to characterize mass transfer into artery, and especially aorta, walls. They have found focal leakage. That is, intense spots of accumulated tracer tend to form rather than (or in addition to, in the case of small tracers) a uniform darkening. Some have used tagged low-density lipoprotein (LDL); others have used Evans Blue albumin or horseradish peroxidase. The peroxidase molecule, while significantly smaller than the 22 nanometer diameter LDL, is, at a diameter of 6 nanometers, far larger than water and is believed to pass through the endothelial layer and into the artery wall far more easily through so-called leaky junctions, or unusually wide gaps between cells, as proposed by Weinbaum (29). For a more extensive review of the literature on spot size studies of mass transfer into artery walls, see Yu (32).

Materials and Methods

All procedures were IACUC approved. The control experiment followed the method of Yu, with chemicals and other materials sourced from the same suppliers, with one modification. As Yu reports (32),

“To prepare the tracer, we dissolve 0.58g NaCl in 100 ml distilled water; then add 0.024 g HRP (for a 300 g rat) in 1mL normal saline. All procedures are IACUC approved. 30 mg Pentobarbital Sodium/kg body weight rat in a 1% solution (100 mg Pentobarbital Sodium in 10 ml of distilled water, shaken gently) by intraperitoneal injection anesthetizes the rat. We shave the rat’s left leg, dissect it and cannulate the left femoral vein and artery… We intubate the rat by attaching its trachea to a rodent respirator… We inject 1ml HRP and 0.5 - 1ml Heparin (5000 units/1 ml, Elkins-Sinn Inc. NJ). At 0.5, 1, 2… min blood circulation, an overdose of Pentobarbital Sodium sacrifices the rat. After flushing with 10ml PBS (Phosphate Buffered Saline; Sigma) and then perfusion-fixing the vessels in situ at room temperature with 1% Glutaraldehyde (Sigma) (1 ml 50% Glutaraldehyde in 50 ml PBS) introduced into the carotid vein and artery and draining from the femoral arteries, we excise the thoracic aorta… We prepare the excised vessels for examination by peeling away the connective tissues and the adventitia, further fix them in 1% Glutaraldehyde for 1 hour [or overnight], rinse with PBS three time to wash out the fixative and run the HRP-DAB reaction to develop the tracer spot. We dissolve 0.045 g DAB in a solution of 5 ml 0.3M Tris (0.182 g Tris (MW=121.1) in 5 ml H₂O) in 25 g distilled water, add 20
μl of 30% $\text{H}_2\text{O}_2$, followed by 1M HCl to adjust the pH to 7.0~7.4 and then place the tissue in it. The DAB solution is changed after 30 min and allowed to continue for another 30 min. The tissue is washed with PBS solution and prepared on slides for en face examination with an Olympus BX51 light microscope. The reaction product is brown, and so the leak is a brown spot. Since HRP can slowly penetrate normal junctions, focal spots coexist with diffuse staining as in (14). We count the number of HRP spots by eye and measure the area of each piece of tissue. We then calculate the leakage frequency” [in spots per square millimeter].

The modification in the experiments reported here is that the rats were given a larger initial dose of pentobarbital anesthetic of 60 mg/kg. In the apoptosis experiment, the rats were given 5 micrograms (ug) per kilogram of body weight of tumor necrosis factor alpha (TNFA) and 0.75 mg/kg of cycloheximide, both purchased from Sigma Aldrich, intravenously through the femoral vein. The rats were also given periodic booster doses of pentobarbital, based on response to paw-pinches tests and breathing rate, to keep them unconscious over the course of this longer experiment. In one experiment reported here, the drugs were injected in 4 equally divided boluses over half an hour. Since that method appeared to cause respiratory distress, it was rejected in the next 2 experiments in favor of continuous infusion, also over half an hour, which seemed to cause less distress. On the advice of another researcher, Dr. Limary Cancel, who had performed similar experiments on aortic endothelial cell monolayers, we allowed the drugs to circulate for 4 hours to take effect. We then performed the standard HRP experiment (see above). The images below are photographs taken with an Olympus consumer-grade digital camera mounted on an Olympus BX51 optical microscope. The magnification of the microscope was 100x and that of the camera's zoom lens 3x. The images were further enlarged in the process of publishing them in this document.

Results and Discussion

We were concerned about the short biological half life of TNFA. Zahn (33) reports it as 5-8
minutes at a dose of 2 ug/kg administered to rats over half an hour and 30 minutes at 10 or more ug/kg. However, we decided to proceed, on the thinking that, though the drug may be eliminated fairly quickly, the damage it does may not be completely repaired in 4 hours.

The images below show control HRP spots at circulation at circulation times of 30, 60, and 120 seconds. The 4 minute experiment was not attempted, because other researchers (4, 32) have reported nearly uniform leakage at that circulation time. Also below are images of HRP spots in apoptosis-induced rat aortas. As the images demonstrate, the staining in the apoptosis-induced samples (figures 2.4, 2.6, 2.8, 2.10) is much fainter than that in the controls (figures 2.1, 2.2, 2.3, 2.5, 2.7, 2.9). Both the spots and the background appear much lighter. It was for this reason that we did not attempt a 30 second experiment; we feared that the spots would not be visible enough to count in order to determine their frequency. We conjectured that a possible cause of this faint staining could be the deteriorating overall health of the animal over the course of the experiment, which took at least four hours longer than the control experiment. During the entire course of the experiment the animal was anesthetized with pentobarbital, a drug whose effective dose (60 mg/kg delivered by intraperitoneal (IP) injection in this study, in line with our Institutional Animal Care and Use Committee’s recommendations) is within a factor of two of its mean lethal dose (120 mg/kg IP (34)). The American Veterinary Medical Association recommends a euthanizing dose of three times the anesthetic dose for rodents (35). The animals' heart and breathing rates were both observed to decrease as the experiment continued, and it is reasonable to assume that blood pressure may have also, which would have decreased the force driving solute into the aorta wall. If this experiment were to be repeated, it might be helpful to introduce the drugs via a tail vein cannulation instead of a femoral cannulation. This is a more difficult technique, but it is far less invasive and, therefore, requires less anesthesia. This might allow data
collection from a healthier animal.

The frequency of the control HRP spots was found to be 0.80 spots per square millimeter of aorta (9 rats) surface, in good agreement with Yu's result of 1.04 (35 rats) (32). In the apoptosis experiments, the frequency was found, in direct contradiction of our prediction, to be a lower 0.46 (3 rats). One possible explanation would be the declining health of the animal treated with anesthetic doses of pentobarbital for a number of hours, as discussed above. Another possible cause is the toxicity of the chemical treatment. Although Zahn (33) reports no toxicity in rats treated with doses of TNFA up to 100 times higher than ours, our dose of cycloheximide was half of the lethal dose (17, 28). Still another has to do with the short half life of TNFA (on the order of minutes). We did not attempt to predict how long it should take for a rat's aortic endothelium to be restored from especially leaky (the condition expected as a result of the chemical treatment) to typically leaky (the undisturbed state, once the chemicals have been cleared and their effects neutralized), but one thing is clear from the value of the half life: after 4 hours, the amount of active drug in the rat is negligible.
Figure 2.1: 30s control HRP spot

Figure 2.2: 30s control HRP spot
Figure 2.3: 60s control HRP spot

Figure 2.4: apoptosis 60s HRP spot
Figure 2.5: 60s control HRP spot

Figure 2.6: apoptosis 60s HRP spot
Figure 2.7: 120s control HRP spot

Figure 2.8: apoptosis 120s HRP spot
Figure 2.9: 120s control HRP

Figure 2.10: apoptosis 120s HRP spot
2.4: Conclusion

In our study, rat aortas exhibited, paradoxically, a lower HRP spot frequency when the rats were subjected to apoptosis-inducing chemical treatment. However, we suspect that this result may be an artifact of the experimental procedure and that the hypothesis that apoptosis leads to a higher spot frequency still warrants investigation.

For future work, in addition to the above suggestion for mitigating the anesthesia effects, there are several ways to test the hypothesis that inducing apoptosis increases spot frequency. One would be to use TNFA in combination with another drug, as shown by Zahn (33) to increase TNFA’s half life. Another would be to use polyethylene glycol-coated TNFA, which Tsutsumi (27) has shown also has a longer half life than native TNFA. A third option, one currently under consideration, would be to use a cytotoxic agent with a naturally longer half life. The anti-tumor drug Paclitaxel is one such drug, and we are planning experiments using it.

Further, it would be useful to run several experiments with only TNFA and several with only cycloheximide. This could help us tease apart their independent effects, both on spot frequency and on overall animal health.
References


9. Han P. The combination of atenolol and amlodipine is better than their monotherapy for preventing end-organ damage in different types of hypertension in rats. Journal of Cellular and Molecular Medicine. 2009 Apr;13(4):726-734.


