Scanning Microscopy

Volume 3 | Number 1 Article 31

3-15-1989

Ranking Corrosion Efficiency: A Latin Square Study on Rat Lung Microvascular Corrosion Casts

Dean E. Schraufnagel
University of Illinois at Chicago

Scanning Microscopy, Vol. 3, No. 1, 1989 (Pages 299-304)
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

0891-7035/89$3.00+.00

RANKING CORROSION EFFICIENCY: A LATIN SQUARE STUDY ON RAT LUNG MICROVASCULAR CORROSION CASTS

Dean E. Schraufnagel

Section of Respiratory and Critical Care Medicine,
Department of Medicine (M/C 787), University of Illinois
at Chicago, P. O. Box 6998, Chicago, IL 60680, U. S. A.
Phone No. (312) 996-3820

(Received for publication November 17, 1988, and in revised form March 15, 1989)

Abstract

Scanning electron microscopy of corrosion casts is an important tool for the study of microvascular structure but few systematic studies on methods of specimen preparation have been reported. This study sought to determine the relative importance of factors involved in corrosion. It compared potassium hydroxide, sodium hydroxide and water as major corrosive agents. It tested the size of the tissue sample, and the use of prealkali autolysis, detergent, and proteolytic enzymes in a Latin square designed study. The main findings were that sodium and potassium hydroxide were better than water ($P<0.0001$) and the longer the corrosion time the better corroded the samples were ($P<0.0001$). Although not a controlled factor, higher room temperature was also associated with better corrosion. The use of proteolytic enzymes, detergent, and warm tap water alone before the alkali treatment did not significantly improve the corrosion in this study, although this does not preclude an effect with another experimental design.

Key words: Histologic techniques, scanning electron microscopy, blood vessels, pulmonary circulation, microcirculation, microcorrosion casting.

Introduction

Corrosion casting is an important tool to study microvascular structure but few systematic studies on the methods for preparation of microvascular casts of the lung for scanning electron microscopy have been reported (Schraufnagel and Schmid 1988a, Schraufnagel and Schmid 1988b). In this study I asked the question what factors improved corrosion? Is potassium hydroxide better than sodium hydroxide? Is the size of the tissue, precorrosion autolysis, detergent, or proteolytic enzymes important? Ideally each factor should be the subject of a separate study, but to conserve resources I performed a single Latin square designed study (Snedecor and Cochran 1980, SAS 1985) that included these variables.

Methods

Fifteen female, 250 g Sprague-Dawley rats were anesthetized with enough pentobarbital to produce profound anesthesia. The abdomen was opened and the caudal vena cava and abdominal aorta were cannulated at the level of the renal arteries. Heparinized saline, warmed to 45°C was infused into the caudal vena cava until the aortic effluent was clear. Fifty milliliters of buffered 10% formalin (Fisher, Chicago, IL) was then injected into the caudal vena cava by a hand held syringe. After about 5 minutes, 15 ml of partially polymerized methylmethacrylate (Mercox, Ladd Research Industries, Burlington, VT) mixed with catalyst was injected through the caudal vena cava to fill the pulmonary vasculature. The animals were placed in a 45°C bath for one hour before the lungs were removed. Incompletely cast lungs were discarded and the treatment was given to a new animal.

To test specimen size, the right lung with its 5 lobes was compared to the left lung with only one lobe (and about half the volume of the right). A random number generator assigned the right and left lung of each animal to a treatment listed in the appendix. The precorrosion solutions were:

1. a detergent, Alconox (Alconox Inc, New York, NY), 30 g/l,

2. a detergent-proteolytic enzyme solution, Terg-A-Zyme (Alconox Inc) containing the same detergent (Alconox 30 g/l) and a *Bacillus subtilis* enzyme (0.15 g/l),

3. a *Bacillus subtilis* enzyme alone (1.5 g/l) (Esparase, Alconox, Inc), and

4. warm tap water alone. The enzyme was provided by the manufacturer who stated that the potencies of the enzymes in Terg-A-Zyme and Esparase were equivalent.

In addition, specimens were placed immediately into the corroding agent that was either 10 N NaOH, 10 N KOH, or water. The precorrosion solutions were warm (about 40°C), but were allowed to cool to room temperature. The cast tissues were sampled starting a day after being placed in alkali by rinsing them and removing 1 mm slices by razor blades and returning the tissue to the alkali. The study ended when no more cast was available or at 45 days if tissue still remained.

All procedures were done at room temperature which was recorded daily. The temperature of the room for the time when the specimen was corroding was averaged and considered in the analysis. Slices were placed in a tea strainer and rinsed for 20 minutes in rapidly flowing tap water. The sections were rinsed with ethanol, air-dried and fastened to aluminum studs with double-sided tape, sputter-coated with a layer of palladium-gold about 20 nm thick and viewed with a JEOL JSM-35C scanning electron microscope.

Judging the adequacy of corrosion was done without knowledge of which group the specimens belonged. For each stud five randomly selected fields were viewed at 20X, 200X, 480X, 2000X and 4000X magnification. Each of the 20 fields was graded. Five was assigned to fields that had cast completely obscured by amorphous, uncorroded material. Four was assigned to fields that had large patches of uncorroded material. Three was assigned to fields that had extensive debris but no cast part was obscured by material more than 10 μm in diameter. Two was assigned to fields with specks of debris but were otherwise clean. One was assigned to clear fields (figures 1-5). The five numbers for each magnification were added. The lower magnifications were weighted by multiplying an order of magnitude for each magnification. The sum for the 20X field was multiplied by 10^5; the sum of the 200X field was multiplied by 10^4; the sum of the 480X field was multiplied by 10^3 and so on. The products of each magnification were then added together. This weighting system caused the higher magnifications to be important only if the lower magnifications were equal. The numbers were then ranked and the distribution of the ranks was analyzed for normality by the Kolomogorov test (SAS 1985). The plan was to then do a multiple regression of these ranks with the factors to be tested. Time was taken into account by using it as a factor in the multiple regression analysis and by multiplying the rank times the day the tissue was sampled. Lower scores meant better corrosion in fewer days. However, neither the ranks nor the ranks-times-days scores were normally distributed, so that the log of the scores was taken. The first regression studied the interaction terms. The interaction terms included combinations of the alkali (sodium, potassium, or water), precorrosion (water, detergent, enzyme, or detergent and enzyme), tissue size (right or left lung) and immediate alkali. All combinations of these terms were considered. The final regression equation deleted the nonsignificant factors (SAS 1985).

Results

The corrosion ranks were not normally distributed because many casts were well corroded and others were completely uncorroded even at the end of the study. The log of the ranks-times-days was normally distributed and used for the regression. However, the log scores and ranks gave the same results.

The main finding was that both potassium hydroxide (partial $R^2 = 0.2075$, $P < 0.0001$) and sodium hydroxide (partial $R^2 = 0.2060$, $P < 0.0001$) were better than water. Not surprising, time was also important ($P < 0.0001$) in producing well corroded casts. The only other variable that was important was the room temperature (partial $R^2 = 0.1378$, $P = 0.0001$). No other variable or combination of variables was significant at the 0.05 level, although they all pointed in the expected direction. The use of a proteolytic enzyme had a partial $R^2 = 0.01$, $p = 0.12$ and was the next most important factor. The total R^2 for NaOH, KOH and temperature was 0.55.

These experiments took place during the winter and the room temperature fluctuated so that the average daily temperature for the specimens varied between 21.0 and 24.5°C.

Discussion

Although different authors have advanced different methods of corrosion (Hodde 1981, Lametschwandtner et al 1984, Schraufnagel 1987, Christofferson and Nilsson 1988), few systematic reports of corrosion have been published. Gannon (1978) suggested that KOH was better than NaOH. Hodde (1981) recommended placing tissue in warm water for 1 to 2 days to begin decomposition and changing the alkali frequently with fresh water. Our samples were changed daily but were not rinsed extensively between alkali changes. Christofferson and Nilsson (1988) noted that no method reproducibly gave good corrosion because of poorly penetrated islands of tissue. They recommended frequent agitation was beneficial, but this was not tested in this experiment. We noted that rats given intratracheal elastase to produce emphysema had casts with less tissue than those receiving saline (Schraufnagel and Schmid 1988c).

As we found previously, viewing casts with the dissecting microscope was a poor method of determining how well tissue was corroded (Schraufnagel and Schmid 1988a, 1988b). Determining that a cast was corroded was not easy with the electron microscope either. Subjective evaluation was poorly reproducible unless the

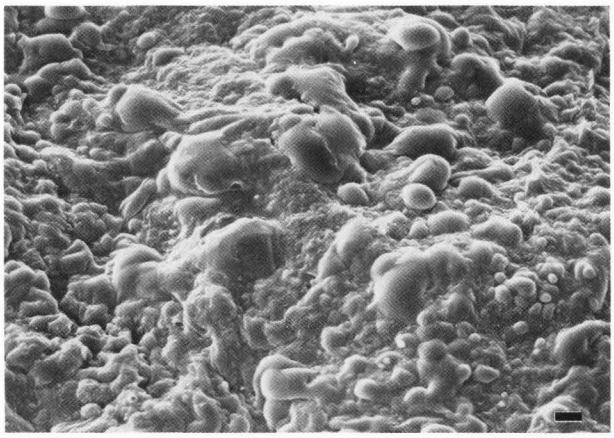

Figure 1. The micrograph would be scored 5 because it is an amorphous mass. Casts are not clearly distinguishable. Bar = 10 μm.

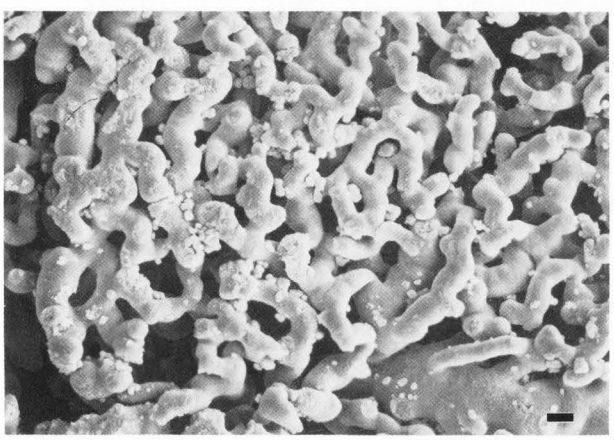

Figure 3. This micrograph would be scored 3 because areas of cast 10 μm or less are obscurred. Bar = 10 μm.

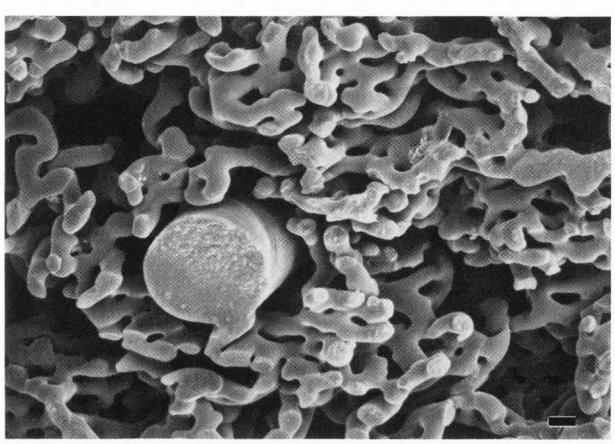

Figure 5. This micrograph would be scored 1. It is free of tissue. Bar = 10 μm.

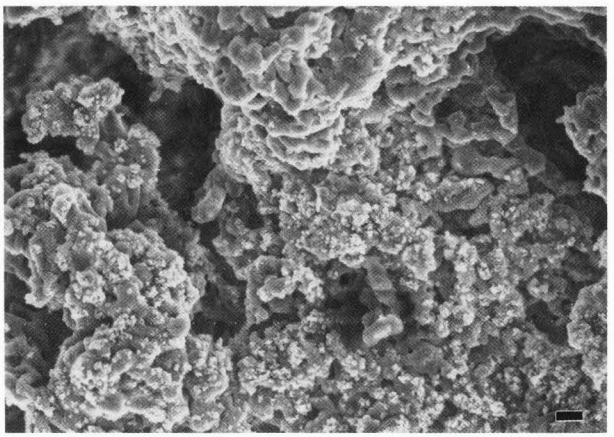

Figure 2. This micrograph would be scored 4 because large areas (> 10 μm) of the cast are covered with undigested tissue, but cast vessels are distinguishable. Bar 10 = μm.

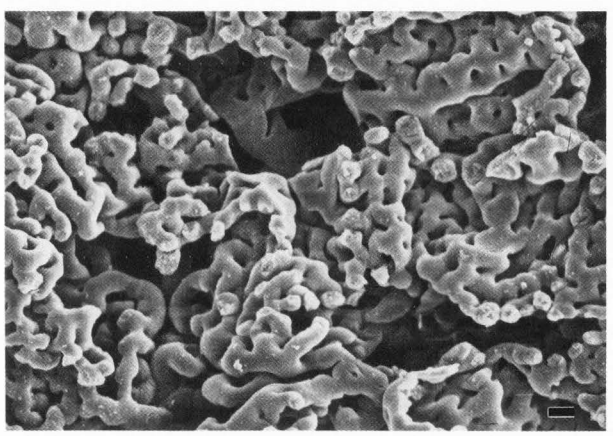

Figure 4. This micrograph would be scored 2 because only scattered debris is present. Bar = 10 μm.

magnification was fixed and the several fields were viewed. The ranking was used to analyze data which by its nature is ordinal. The weighting made higher magnifications important only if the lower magnifications did not distinguish how well the tissue was corroded. Usually, if the higher magnifications were clear of tissue, the lower magnifications also were. However, even ranking was not free of drawbacks. The ranks were not normally distributed because once specimens were corroded their corrosion score did not change. Most of the tissue in water did not corrode. Taking the log of the ranks made their distribution normal to allow the multiple regression. Fortunately, the ranks and the logs of the ranks gave the same answer.

Another problem was the uncertainty that debris was the result of poor corrosion instead of poor rinsing. Rinsing was not tested in this study. In previous studies we rinsed specimens overnight before

mounting, but because so many small specimens were taken, the increased time and risk of losing small specimens did not allow extensive rinsing. It appears that fewer excellent specimens were seen in this study than in our other studies.

Although most specimens that were put in hydroxide produced clean casts by 1 week, it was disappointing that well corroded casts were not uniformly obtained in short times. A difference between potassium hydroxide and sodium hydroxide was not apparent. The value of preincubation in water, detergent or proteolytic enzyme solutions was not demonstrated even though there is good anecdotal information on and theoretic advantage to these procedures. It was surprising that warmer room temperature was a more significant factor than the precorrosion variables. During the study we had days of central heating malfunction so that the room temperatures were elevated. However, since this was not a controlled factor, it needs further study. Murakami (1978) among others has advocated using higher temperature

The lack positive of findings of the precorrosion variables may be linked to several factors. The most important may be that the Latin square design does not have as much power as individual studies with larger numbers of samples, although this indicates that the effect of the prealkali variables is less than anticipated. However, a factor such as room temperature, insufficient rinsing or agitation, or too strong or too weak alkali may blunt the contribution of the precorrosion factors.

Acknowledgement

I thank Laura Kadzie, Dr. Ketan Patel, and the Electron Microscopy Facility of the Research Resources Center, University of Illinois at Chicago for help and the equipment necessary to conduct this study.

References

Christofferson RH, Nilsson BO. (1988). Microvascular corrosion casting with analysis in the scanning electron microscope. Scanning **10**:43-63.

Gannon BJ. (1978). Vascular casting. In: Hayat MA (ed); Principles and Techniques of Scanning Electron Microscopy. Biological Applications; Volume 6. Van Nostrand-Reinhold, New York, 170-193.

Hodde KC. (1981). Cephalic vascular patterns in the rat. University of Amsterdam, Amsterdam, 17.

Lametschwandtner A, Lametschwandtner U, Weiger T. (1984). Scanning electron microscopy of vascular corrosion casts - technique and applications. Scanning Electron Microsc **1984**; **II**:663-695.

Murakami T. (1978). Methyl methylmethacrylate injection replica method. In: Hayat M, (ed); Principles and Techniques of Scanning Electron Microscopy. Biological Applications 2, Van Nostrand-Reinhold, New York, 159-169.

SAS Institute. (1985). SAS User's Guide Version 5: Statistics. SAS Institute, Cary, NC.

Schraufnagel DE. (1987). Microvascular casting of the lung. A state-of-the-art review. Scanning Microsc **1**:1733-1747.

Schraufnagel DE, Schmid A. (1988a). Microvascular casting of the lung: vascular lavage. Scanning Microsc **2**:1017-1020.

Schraufnagel DE, Schmid A. (1988b). Microvascular casting of the lung: effects of various fixation protocols. J Electron Microsc Techn **8**:185-191.

Schraufnagel DE, Schmid A. (1988c). Capillary structure in elastase-induced emphysema. Am J Pathol **130**:126-135.

Snedecor GW, Cochran WG. (1980). Statistical methods. Seventh Ed. Iowa State U Press, Ames 269-273.

Discussion with Reviewers

R. H. Christofferson: Do you think "autolysis" is a suitable term for immersing formalin perfusion-fixed tissue in water for 45 days or less? Could the low significance of precorrosion treatment be explained by denaturation of tissue proteins due to fixation?

Author: Autolysis may not have been given a fair chance and perhaps this term should not even have been used because the tissue only soaked in water overnight at room temperature. The use of formaldehyde would be expected to delay tissue maceration.

R. H. Christofferson: What is the reason for idiosyncrasies such as one virtually uncorroded specimen in a batch of otherwise corroded specimens? Could you have missed an important confounding factor such as fixation, agitation, rinsing or temperature?

Author: I do not know the reason for the differences in digestion of tissues treated alike. One animal (# 815) had tissue present after weeks of corrosion. Although experimental error is possible, I could not find any on rechecking. Interanimal differences or polymerization of tissue is also possible. Removing this outlier from the analysis did not change the results. The idea that areas of tissue have less contact with corrosion agents (Christofferson and Nilsson 1988) is an explanation. The other factors you mentioned could also be important.

R. M. Albrecht: Perhaps higher concentration of enzyme, longer times, higher temperature, or alternating hydroxide with enzyme may improve digestion.

Author: I agree. Temperature, agitation, rinsing, and different concentrations of the enzymes are worthy areas to investigate.

R. H. Christofferson: To conserve resources it is better not to increase complexity -- why did you use a commercial detergent and detergent-enzyme solution of unknown composition and quality from batch to batch instead of a well-defined detergent and enzyme?

Author: I wanted material that was readily available. The enzymes of *B. subtilis* are much cheaper than pure elastase or collagenase. The scientists from

Appendix: Scheme of applying treatments to specimens

Animal	Lung	Alkali	Pretreatment	Rank times days	Final Rank
818	Right	NaOH	Terg-A-Zyme	88	17
	Left	NaOH	Alconox	110	2
822	Right	NaOH	Esparase	67	1
	Left	NaOH	Water	226	6
814	Right	Water	Immediate alkali	1532	24
	Left	KOH	Terg-A-Zyme	195	5
821	Right	KOH	Alconox	191	3
	Left	KOH	Esparase	448	10
820	Right	KOH	Water	194	4
	Left	KOH	Immediate alkali	321	7
825	Right	Water	Terg-A-Zyme	1577	25
	Left	Water	Alconox	2178	29
817	Right	Water	Esparase	1209	22
	Left	Water	Water	1244	23
813	Right	NaOH	Immediate alkali	782	14
	Left	NaOH	Terg-A-Zyme	1095	21
816	Righ	NaOH	Alconox	355	8
	Left	NaOH	Esparase	441	9
812	Right	NaOH	Water	841	15
	Left	NaOH	Immediate alkali	702	12
815	Right	KOH	Terg-A-Zyme	1626	26
	Left	KOH	Alconox	2050	27
824	Right	KOH	Esparase	782	13
	Left	KOH	Water	698	11
826	Right	KOH	Immediate alkali	855	16
	Left	Water	Terg-A-Zyme	925	18
819	Right	Water	Alconox	1006	20
	Left	Water	Esparase	999	19
823	Right	Water	Water	2525	30
	Left	Water	Immediate alkali	2122	28

The prealkali treatments were detergent (Alconox), a proteolytic enzyme (Esparase) or both detergent and proteolytic enzyme (Terg-A-Zyme). The immediate alkali could be either KOH, NaOH or water. The effect of larger tissue sample was tested by using the right and left lungs. The right lung is about twice as large as the left.

Alconox assured me that enzyme activity of Terg-A-Zyme and Esparase at the concentrations used were equivalent.

R. H. Christofferson: Was important information obtained from partially corroded casts?

Author: I did not address that question here, but other studies have (Wang and Wei 1976, Wang and Ying, 1977, Schraufnagel 1987). Partial digestion studies have a great deal of information to give for the right research questions.

Christofferson: Were extravasations of casting medium discriminated from undigested material in the grading?
Author: Occasionally this was a problem, but probably not as often in the lung as in other organs because of the easily recognizable air-filled alveoli. Material that does not go away regardless of the corrosion time may also be alkali-induced polymerization of aldehyde. Formaldehyde or glutaraldehyde that penetrates the tissue may be polymerized by the strong alkali. The plasticized tissue is then protected from corrosion.

R. H. Christofferson: In my own experience corrosion is most efficient the first two weeks and there is little point in continuing corrosion after that. Do you have similar experiences?
Author: I plotted the digestion score against time and found continuous improvement with time, but there was a great deal of scatter. I do agree we should have a recipe that gives consistently clean casts within 2 weeks.

R. M. Albrecht: Could you tell how quickly your best preparations produced clean casts?
Author: I ranked the specimens by the tissue digestion score and found good casts produced after a day of digestion; most of the top scores were in the first week. Essentially all of the water scores were terrible; delayed versus immediate alkali (as I tested it) was of no consequence. I doubt the detergent has any effect. The enzyme may, but I did not show it.

R. M. Albrecht: We have used much reduced concentrations of NaOH (2% to 7%) at temperatures of 45°C to produce cleanly digested casts of rat lung by 1 to 3 days while higher hydroxide concentrations appear to require additional time. Have you had similar experience? Could the higher concentration of hydroxide produce chemical changes especially in lipids that actually increase the resistance of the tissue to alkali?

Author: I have not systematically used lower alkali concentrations. Lower alkali concentration may avoid the polymerization effect mentioned above. The higher temperature is also likely to be important.

Additional References

Wang NS, Wei F. (1976). Scanning electron microscopy of capillary and elastic fiber networks in alveoli. Scanning Electron Microsc **1976; II**:233-238.

Wang NS, Ying WL. (1977). A scanning electron microscopy of alkali-digested human and rabbit alveoli. Am Rev Respir Dis **115**:449-460.