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ANATOMY AND MORPHOMETRY OF MYOCARDIAL CAPILLARIES STUDIED WITH VASCULAR CORROSION CASTING AND SCANNING ELECTRON MICROSCOPY: A METHOD FOR RAT HEART

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Abstract

The present paper describes a procedure for preparing vascular corrosion casts of rat myocardial microvasculature. Essential components of the procedure include: partial "self clearing" of the heart in vitro; cardiac arrest by infusion of KCl; retrograde aortic root infusion of Mercox-Sevriton casting resin; KOH digestion of ventricular tissue; and desiccation and mounting of casts for scanning electron microscopy. About 50% of rats yielded complete casts. Vasculature closely paralleled muscle fiber orientation. Capillary beds characteristically exhibited branching, many intercapillary cross bridges, and occasional coiling. Average capillary cast diameter (5.6 μm) and intercapillary distance (15 μm) are comparable to results from in vivo studies. From preliminary calculations, vascular volume represents about 10% of the ventricular walls. These data indicate that vascular corrosion casts may be useful in the analysis of pathologic states and in determining the role of potential therapeutic interventions.

KEY WORDS: rat myocardium, capillaries, vascular corrosion casting, scanning electron microscopy, morphometry

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Introduction

The exchange of oxygen and nutrients in the myocardium depends on the intimate relationship between cardiac myocytes and their capillaries. In several pathological states such exchange is compromised by alterations in the myocyte. For example, in left ventricular hypertrophy secondary to hypertension or valvular aortic stenosis capillary density is lower than in normal hearts (see discussion by Hudlicka, 1984). If a means for studying the three-dimensional anatomy of the myocardial microvasculature was available, it might be possible to measure microvascular changes in pathologic states and their response to various therapeutic agents and procedures. The refinement of vascular corrosion casting techniques (see review by Lametschwandtner et al., 1984) and the application of scanning electron microscopy (SEM) to corrosion casting (Murakami, 1971) permit viewing of three-dimensional myocardial microvasculature with a degree of resolution not possible by other methods. If the method faithfully depicts the in vivo state, microvascular corrosion casting can be a powerful tool in cardiology.

In the present paper, we (1) describe a useful procedure for the preparation of rat myocardial microvascular casts, (2) provide evidence that the resulting casts do reflect the in vivo state, and (3) indicate with preliminary data that such corrosion casts may be used for quantitative measurements. Portions of this study have been presented in abstract form (Douglas et al., 1985; Hossler et al., 1985).

Materials and Methods

Microcorrosion casting procedure

Sprague-Dawley rats (male or female, 200-500g) were anesthetized with pentobarbital (13-30 mg) and anticoagulated with heparin (2000-5000 U) intraperitoneally. After 45-60 min, the beating hearts with great vessels attached were removed through a midsternal incision, immersed in warm (30°C) 0.9% NaCl containing heparin (15-20 U/ml) and allowed to beat spontaneously for several minutes. A flared canulla (usually 1.14 mm i.d.) attached to a syringe needle (18 ga) was then inserted retrograde into the ascending aorta

and secured with a ligature. The heart suspended in saline was allowed to beat several more minutes until fluid filled the cannula and was ejected from the Luer fitting of the needle. A syringe was then attached to the needle and the heart was flushed with gentle manual pressure on the syringe first with 10 ml of heparinized 0.9% NaCl and then with 10 ml isotonic KCl. The needle with heart attached was then fitted to a three-way valve suspended over a catch basin. In all steps care was taken to avoid introducing air into the system. Each heart was then perfused an additional five minutes with 0.9% NaCl (30°C) at a monitored pressure of 80-100 mmHg.

Mercox resin (Ladd Research Industries, Burlington, VT) or Mercox resin mixed with Sevriton dental sealant (Dentsply Ltd., Weybridge, Surrey, England) was then infused at the same pressures through the remaining port on the three-way valve. Resin mixtures were prepared immediately before use and consisted of Mercox resin plus catalyst (5 ml:0.15ml) or Mercox resin plus Sevriton plus catalyst (4ml:1ml:0.2ml). Infusion of the resin was continued until the onset of polymerization (usually 5 min). Hearts were then immersed in warm water (about 50°C) for 30-60 min to complete polymerization. Tissue removal was accomplished by repeated maceration in 20% NaOH or KOH (usually 48 h) followed by several rinses in distilled water (usually 24 h each). After a thorough rinsing in distilled water heart casts were air dried or critical point dried (model E 3000, Polaron, Watford, England), sectioned with a heated wire apparatus, affixed to stubs with silver paste, sputter coated (model Desk-1, Denton Vacuum, Inc., Cherry Hill, NJ) with gold-palladium, and viewed and photographed with a scanning electron microscope (model S-430, Hitachi Scientific Instruments, Inc., Mountain View, CA). Measurements were made from micrographs.

Calculation of vascular volume

For vascular volume calculations, cast hearts (before maceration) were sliced transversely with razor blades into apical, middle, and upper thirds and each third was divided into right ventricular, left ventricular, and interventricular segments. Each segment was blotted dry, weighed, and then macerated as described above. Only hearts or segments of hearts which appeared at least superficially to be completely perfused with resin and subsequently showed excellent casts by SEM, were used for vascular volume calculations. After drying, casts of the various myocardial segments were again weighed and the proportion of the myocardium which was vascular space was calculated. From polymerized resin blocks densities of 1.18 and 1.20 g/ml were calculated for Mercox and Mercox-Sevriton, respectively. Myocardial density was assumed to be 1.05 g/ml (Helak and Reichek, 1981). Total segment volume equaled cast volume plus tissue volume, cast volume was calculated from cast weight and resin density, and tissue volume was calculated from tissue weight and density.

Estimation of resin viscosity

Relative viscosities of resin mixtures at 30°C were estimated by determining flow rates in glass capillary tubing (2mm i.d.) using aqueous glycerol solutions as standards. Although the viscosity of Mercox is reported as 20-30 cps by the manufacturer, the measured values varied somewhat from one shipment to the next and were often considerably higher than reported. However, the addition of 20% Sevriton reduced the viscosity by about 50% in all cases. Increasing the percentage of Sevriton above 20% prevented polymerization.

Results

Routinely about 50% of the hearts yielded complete casts of essentially all of the ventricle myocardial vasculature. All hearts yielded at least some areas of excellent vessel filling. In some hearts, although the epicardial vessels and subepicardial myocardium appeared well perfused, endocardial or subendocardial capillary beds were incompletely cast. When incomplete casts were obtained, the areas most resistant to complete filling were the atrial appendages, and the left ventricular apex. A prediction of the final quality and completeness of a vascular cast of a given heart could be made within the first minute following initiation of resin infusion. In every instance when high quality, complete casts were obtained, rapid and very uniform blanching of all regions of the initially tan myocardium occurred, yielding a subtly striated, whitish surface. The surface striations clearly followed the changing, sometimes swirling patterns of the myocardial fibers. Typically, we observed very little resin in the left ventricle lumen, indicating that the aortic valve had remained competent during the resin infusion.

In developing the casting procedure several steps were especially important. When infusion pressure exceeded 80-100 mmHg, the ventricular lumen was sometimes distended with resin, presumably due to aortic valve failure, which apparently interfered with myocardial vascular casting. Arresting the heart with KCl also enhanced vessel perfusion. The frequency of complete casts was improved when the viscosity of Mercox was reduced by adding 20% Sevriton. Because Mercox-Sevriton casts were susceptible to collapse during air drying, critical point drying was used with this mixture to minimize damage to the delicate, three-dimensional architecture of the capillary beds. Finally, we found that allowing the cannulated heart to beat in vitro while suspended in warm heparinized saline was helpful for at least two reasons. First, the heart initiated its own saline irrigation by drawing saline in through the severed pulmonary veins (thus mimicking somewhat the in vivo situation); and second, because the aortic cannula was filled by the heart's own pumping action, the chance of introducing air bubbles into the perfusion system was reduced.

For scanning electron microscopy and morphometry, regions near the subendocardial and subepicardial surfaces of both ventricles were used. Excellent capillary filling was indicated by the presence of very few blind ended vessels. Characteristically, capillary beds paralleled muscle fiber orientation (Figures 1 and 2), exhibited many cross bridges, usually forming "H"

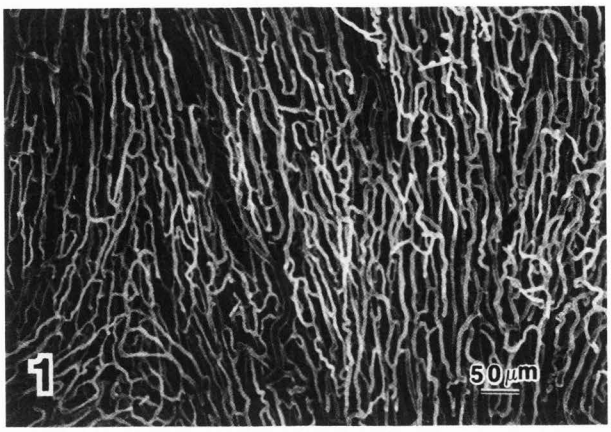

Fig. 1 Overview of vascular corrosion cast of capillary bed of subendocardium of rat left ventricle.

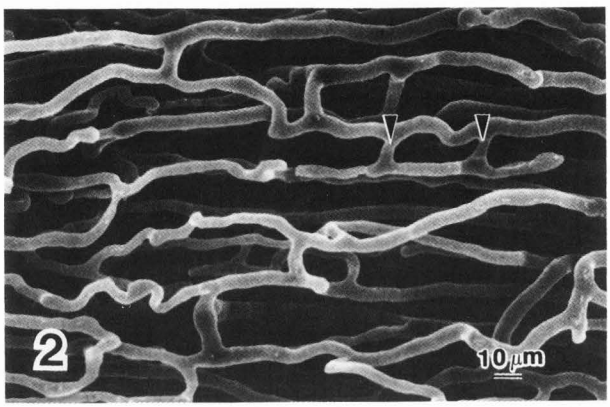

Fig. 2 Details of vascular corrosion cast of capillaries. Note numerous cross bridges often forming "H" patterns (arrowheads).

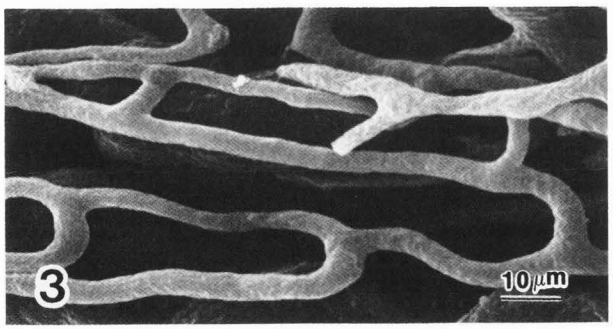

Fig. 3 Details of vascular corrosion cast of capillary branching.

patterns (Fig. 2), and branched frequently, often in a cascading fashion (Figs. 2 and 3). Coiling was also sometimes observed (Fig. 4). Casts of larger vessels exhibited well defined, endothelial nuclear impressions (Fig. 5)

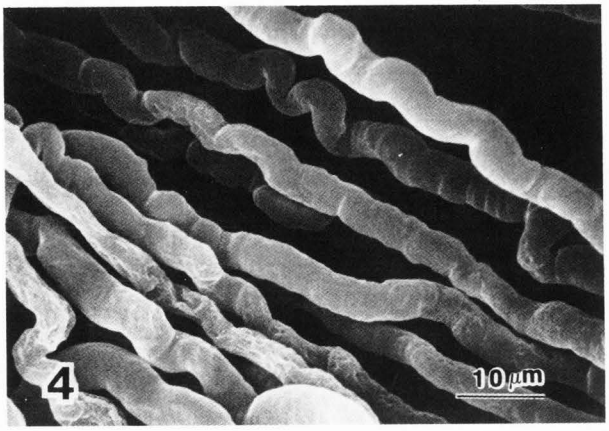

Fig. 4 Details of vascular corrosion cast of capillary bed showing coiling.

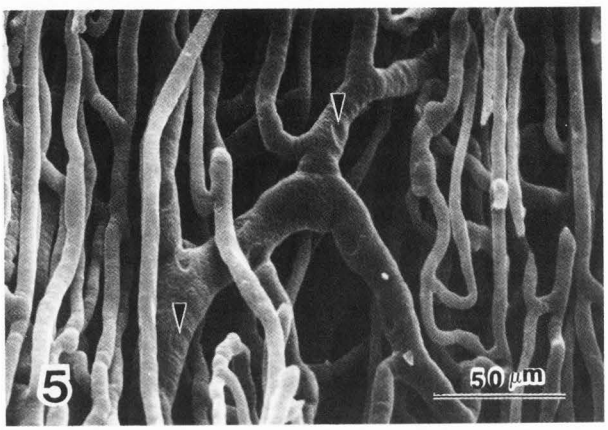

Fig. 5 Details of vascular corrosion cast of capillary bed showing a venule exhibiting prominent endothelial nuclear impressions (arrowheads).

Capillary diameters and intercapillary distances (Table 1) were similar in subendocardial and subepicardial regions. In order to minimize the effects of cast distortion due to drying or handling, intercapillary distances were measured, whenever possible, at sites of capillary cross bridges (i.e., "H" configurations).

Similarly, we observed very little difference between various regions of the ventricular myocardium with regard to vascular volume. In all cases vascular volume represented about 10% of the myocardium (Table 2).

Discussion

Requirements for successful casting of myocardial microvasculature were generally similar to those for vascular corrosion casting of other organs as superbly reviewed by Lametschwandtner et al. (1984). However, several characteristics of myocardial vasculature are unique. Myocardial perfusion occurs primarily during diastole and is impeded by myocardial contraction and by elevated

intracavitary pressures. In the present procedure each heart was arrested in diastole by retrograde isotonic KCl perfusion. Our earlier casting efforts were thwarted when using perfusion pressures of 160-180 mmHg, measured externally, which created aortic valve incompetence and left ventricle cavitary distention. Therefore, we used perfusion pressures of 80-100 mmHg or less. Many of our best casts were obtained when little resin was found in the left ventricular lumen, supporting the contention that aortic valve incompetence and abnormal intracavitary pressures adversely affect myocardial perfusion. Of course, some resin is always found in the left ventricular lumen after casting, presumably due to the presence of the Thebesian vessels. That the perfusion pressures used were not excessive is supported also by the fact that extravasated resin was rarely observed by SEM in our casts.

It is generally agreed that the high viscosity of many casting resins impedes perfusion. Reduction in the viscosity of Mercox (reported as 20-30 cps by the manufacturer) by about 50% by the addition of 20% Sevriton enhanced our casting efficiency. The successful use of Sevriton combined with Batson's medium was previously reported by Nopanitaya et al. (1979). A large variety of low viscosity resins are becoming available and these merit investigation as potential corrosion casting media. The acrylic resin L.R. White (8-10 cps), for example, was used quite successfully by Sage and Gavin (1984) to identify functional capillaries in rat myocardium.

Anatomical features of rat myocardial capillary beds described here are quite similar to those observed previously with other procedures. Capillary density is very high compared with most other tissues (Hudlicka, 1984), and adjacent capillaries are generously interconnected by cross bridges which often form "H" patterns (e.g. rat: Potter and Groom, 1983; cat: Phillips et al., 1979; dog: Bassingthwaighte et al., 1974; Anderson and Anderson, 1980 and 1981; Lametschwandtner and Mohl, 1984; Kajihara et al., 1985; rabbit: Irino et al., 1982; and human: Izumi et al.,1984). The intercapillary distance and capillary diameter measurements we made are similar to those made on other species using different procedures, but most importantly are nearly identical to those made on rat myocardium in vivo (Table 3).

In the present study vascular corrosion casting was used for the first time (to our knowledge) to estimate vascular volume of a tissue. However, the vascular volumes calculated here for ventricular myocardium (about 10%) must certainly be considered minimal values for a number of reasons. First, it is very difficult to obtain 100% filling of capillaries with resin even with the best casts. This is due in part to resin viscosity but also to the fact that many capillaries are held in reserve and are not normally perfused in vivo except under extreme O_2 demand (Henquell and Honig, 1976; Henquell et al., 1977). Secondly, in handling casts during maceration, washing, weighing, and mounting, invariably some of the fine capillary beds are broken and lost. Nevertheless, the use of vascular corrosion casting for quantitation of vasculature is a useful and valuable application of this technique and merits refinement and development.

In summary, a procedure is described here for the efficient preparation of transmural casts of rat myocardial microvasculature. That the casts closely approximate the true three-dimensional vasculature is verified by comparison with results from in vivo studies. In addition, evidence is provided that vascular corrosion casting can be employed for quantitative measurements. These results should provide a basis for the analysis of pathologic states and the microvascular response to potential therapeutic interventions.

Acknowledgements

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Table 1. Capillary Measurements*

Heart Region	Capillary diameter (μm)	Intercapillary distance (μm)	N
Subepicardium	5.7 ± 1.7	15.0 ± 5.3	190
Subendocardium	5.5 ± 2.0	14.8 ± 4.4	164

*Measurements were made from electron micrographs of vascular casts of regions from right and left ventricular walls chosen at random from five hearts. Values are means ± S.D.

Table 2. Determination of Vascular Volume*

Heart region**	Vascular volume (% of myocardium)	N
Right ventricular free wall	10.01 ± 1.06	17
Interventricular septum	8.45 ± 3.08	10
Left ventricular free wall	9.12 ± 2.98	18

*Only sections of complete heart casts were used for measurements. See Materials and Methods. Values are means ± S.D.
**In some cases all three regions were taken from the same heart.

Table 3. Comparison of Myocardial Capillary Measurements in Various Species

Species	Conditions of measurement	Capillary diameter (μm)	Intercapillary distance (μm)	Reference
Dog	Silicone perfusion	5.6	17.5 - 19	Bassingthwaighte et al., 1974
Dog	Corrosion casting	2.5 - 7	7 - 20	Anderson and Anderson, 1980
Rabbit	Corrosion casting	5 - 8	10 - 20	Irino et al., 1982
Rat	Corrosion casting	5.14	—	Potter and Groom, 1983
Dog	Corrosion casting	2.5 - 6	10	Lametschwandtner and Mohl, 1984
Dog	Corrosion casting	4 - 8	—	Kajihara et al., 1985
Rat	Corrosion casting	5.6	15	present study
Rat	in vivo	5.4	—	Henquell et al., 1976
Rat	in vivo	—	14.21	Henquell et al., 1977
Rat	in vivo	6	18.7	Steinhausen et al., 1978

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Discussion with Reviewers

A.C. Nelson: We know that critical point drying and air drying produce different levels of shrinkage. Since you used both methods, how do the two methods affect the observations?
Authors: By reducing surface tension, critical point drying helped prevent collapse of the casts, i.e. the fine capillary casts did not adhere together, and thus the casts retained their natural, three-dimensional configurations and were easier to examine. Measurements of vascular volume, capillary diameter, and intercapillary distance were unaffected by the drying procedure. Recall that intercapillary distances were measured at sites of cross bridging (see Methods). Polymerized resin is apparently much less sensitive to shrinkage than tissue.

A.C. Nelson: Why did you not use chemical fixation in the preparation of the hearts?
Authors: In our experiments aldehyde fixation reduced casting efficiency and quality (perhaps by reducing the natural flexibility of the myocardium necessary for normal perfusion). The use of fixation prior to corrosion casting was discussed at length at SEM/86 meeting and most researchers felt it neither enhanced nor reduced casting quality. The consensus was that fixation was often an extra, unnecessary step in the procedure.

A.C. Nelson: If plastic frequently occurs in the left ventricular lumen, what error would this cause in volume measurement?
Authors: None. Most plastic in the left ventricle of well cast hearts was in the form of free resin droplets which did not distend the lumen. In the few cases of aortic valve incompetence or where perfusion pressure was too high, the left ventricular cavity was distended with resin, the myocardial vasculature was obviously not filled and the cast was discarded.

A.C. Nelson: Do you have data comparing Mercox with Mercox-Sevriton casting?
Authors: No, except that we obtained high quality casts from 30-40% of the rat hearts using Mercox, and from 50% of the rat hearts using Mercox-Sevriton.

S.J. Phillips: Figure 1 is described as a survey micrograph of subendocardium of the rat left ventricle. Would you comment on the paucity of larger caliber vessels in this region. Perhaps the rat differs from larger mammals in the incidence and caliber of subendocardial vessels often seen in this region.
Authors: Figure 1 was chosen because of the emphasis on capillaries in this paper. We suspect the rat is similar to other mammals with regard to larger caliber vessels in this region although we have thus far concentrated our observations on the capillaries.

S.J. Phillips: Your comments on the importance of pressure limits on perfusion are interesting. How physically did you measure pressure in your system?
Authors: Pressure was measured with a mercury manometer attached to the aortic cannula about 5 cm from the heart. While this tells us little about the actual intravascular pressure of the myocardium, it does permit reproducibility among experiments.

B.G. Anderson: You have stressed the excellent capillary filling which occurs when using your technique. I would like very much to see a figure included which demonstrates the density of capillaries in the full thickness of the wall. I believe this would strengthen your position considerably.
Authors: Your suggestion is appreciated. Figure 6 shows a full cross-section through the right ventricular free wall in the middle third of the rat heart. Note the capillary density throughout, and the larger caliber vessels of the epicardium and myocardium.

B.G. Anderson: You say that "only hearts or segments of hearts which appeared at least superficially to be completely perfused with resin and subsequently showed excellent casts by SEM were used for vascular volume calculations." I am concerned about the bias introduced into what is supposed to be a quantitative analysis using this method. The consistency of results from vascular casts is certainly a concern of all of us involved in the use of this technique; we do not have a method by which we get a complete filling of all vessels every time. Could you elaborate on how you propose to use the technique in analysis of pathologic states without being influenced by the bias of your sample selection process?
Authors: Your point is a valid one and we share your concerns. This technique will likely not be useful in studying pathologic states where gross vessel blockage is involved, but it could be quite useful in investigating the effects on myocardial microvasculature of hypertension, diabetes, and cardiac hypertrophy, for example. We agree that one must proceed with such quantitative measurements with caution. However, vascular corrosion casting procedures which offer the opportunity to obtain morphological as well as quantitative information are extremely important and should be pursued.

B.G. Anderson: You suggest that the capillaries which are functionally closed at the time of perfusion are not filled by the casting media. Have you definitively demonstrated that there are many capillaries held in reserve after resin perfusion?
Authors: We have not determined the percent of functional versus reserve capillaries. Our suggestion was based on the estimations of reserve capillaries by Henquell and Honig (1976) and Henquell et al. (1977), and on the estimation of functional rat myocardial capillaries using resin filling by Sage and Gavin (1984). The latter authors used low viscosity L.R. White resin and reported that 95.2% of the capillaries appeared functional after procaine treatment, whereas only 62% allowed the passage of resin before treatment.

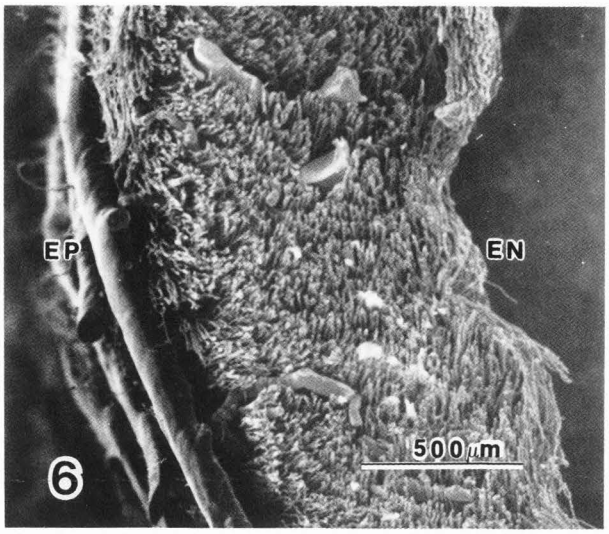

Fig. 6 Vascular corrosion cast of full cross-section of right ventricular free wall in the middle third of the rat heart. EP, epicardial surface; EN, endocardial surface.

H. Kajihara: It is very interesting to use low viscosity resin for the vascular cast. From my experiences, however, it is very difficult to determine whether the filling of vascular beds with resin is enough or not, even if low viscosity resin is used. Moreover, capillary casts are frequently dislocated and destroyed near the cut surface during maceration, washing, and drying. Therefore, measurement of vascular diameter, intervascular space, and vascular density must be carefully made. You obtained good results and discussed well these problems.
Authors: Thank you for your comments.