

## The Ultrastructural Effect Of Bilirubin And Other Plasma Factors On The Basilar Artery In Dogs

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**Abstract :** Vasospasm is the most important factor affecting morbidity and mortality in patients with subarachnoid haemorrhage. Many studies have been done and many factors found to affect vasospasm. The aim of this study is to clarify the factors by observing the ultrastructural effects of plasma and bilirubin on the basilar artery in dogs. Thirteen dogs of 13 – 17 kg were used in three groups , using the suboccipital approach to group A (n:4) 5 ml of 10 day incubated blood containing plasma and erythrocytes

to group B (n:5) 6 ml of one-day incubated autologous blood containing erythrocytes and bilirubin in concentrations equal to that of 10 – day incubated blood, and to group C (n:4) 5 ml saline were administered into the cisterna magna . At the end of 10 days the animals were sacrificed and basilar arteries were evaluated histologically. Morphological studies showed the presence of other factors in plasma which cause vasospasm apart from bilirubin.  
**Key Words :** Bilirubin, Cerebral vasospasm, Plasma

### INTRODUCTION

In cases of subarachnoid haemorrhage resulting from ruptured aneurysms the prognosis is closely related to cerebral vasospasm. There are different points of view concerning the pathogenesis of cerebral vasospasm (3,4,6,7,9). In morphological studies it has been shown that pathological changes occur in all layers of the arterial wall (3,4). It has been put forward that the prominent morphological changes in the cerebral vessels are not only under the direct influence of bilirubin, but that free fatty acids and other factors in plasma are also concerned (3). This study was undertaken to investigate the effects of bilirubin and plasma on cerebral vessels.

### MATERIAL AND METHOD

During this study 13 dogs weighing 13 to 17 kg. were used. 60 ml blood was drawn from each dog under sterile conditions, 20 cc of which was diluted in 1 : 10 ratio with 3,8 % sterile sodium citrate. The rest of the blood was put into 4 test tubes (10 cc in each tube), and washed with heparin.

Leucocytes were decomposed by the Ficcoll–Paque technique (7) and platelets by centrifugation for 15 min (1500 G). Erythrocytes and plasma were put into test tubes, incubated at 37°C for 10 days for the first group (n:4) and for 1 day at 37°C for the second group.

Total bilirubin was measured in the other four tubes with the derivative spectrophotometric technique and albumin with the brom cresol green (BCG) technique at the 3rd, 7th and 10th days and incubated at 37°C under sterile conditions (2,10). Bilirubin was also obtained by extraction of the urine of a patient with obstructive jaundice.

Pulse, blood pressure, temperature and weight were recorded in all animals postoperatively. Sodium thiopental diluted 50 mg/cc injected in a dose of 20mg/kg by the I. V. route. At this dose respiratory depression and full anaesthesia was maintained. Room air was ventilated to all animals.

A small craniotomy was performed at the posterior wall of the foramen magnum. Bilirubin in amounts equal to the amounts of bilirubin in 10 days

incubated was given intracisternally to the first and second groups of animals as below:

First group; 5 cc of the blood that had been incubated for 10 days (2.5 cc erythrocyte + 2.5 cc plasma), second group; 6 cc of blood that had been incubated for 1 day (2.5 cc erythrocyte + 2.5 cc plasma + 1 cc 20 % bilirubin solution), control group 5 cc of saline were injected at 37°C. All dogs were sacrificed on the 10th postoperative day and the basilar arteries were excised for light and electron microscope examinations.

**RESULTS**

**i. Biochemical changes :**

In the blood incubated at 37°C under sterile conditions the bilirubin levels were 0.20  $\pm$  0.03 mg/dl at day 0, but increased to 7.75  $\pm$  0.73 mg/dl at the 10th day. The difference between bilirubin levels at 0 and 10th days was statistically significant ( $p < 0.001$ ) (Figure 1).

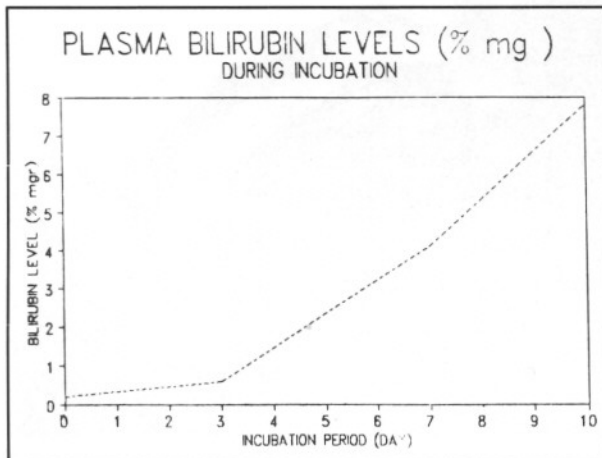


Fig. 1 : Graph of total bilirubin concentrations in serum from the first to the 10th day of blood incubation ( $p < 0.001$ ).

The albumin level decreased from 3.04  $\pm$  0.33 g/dl to 2.67  $\pm$  0.37 g/dl in the same period. This was statistically slightly significant ( $p < 0.05$ ) (Figure 2).

**ii. Light microscope changes :**

Luminal narrowing, endothelial cell swelling, thickening of the medial layer and increase in internal elastic membrane folds were seen in the first group (Figure 3a). In the second group luminal narrowing and increase in internal elastic membrane folds were less than in the first group, but showed difference compared with the control group (Figure 3a,3b,3c).

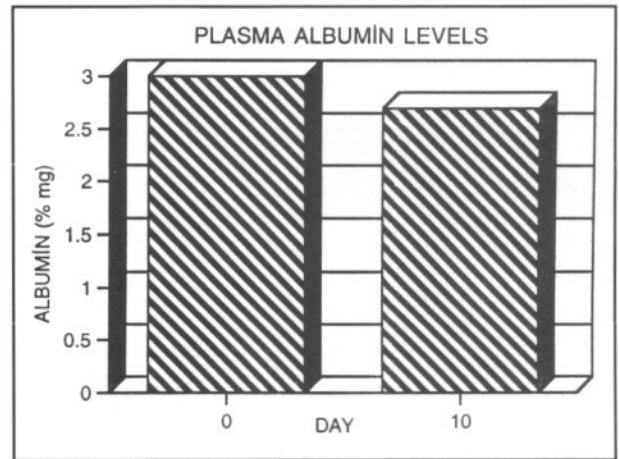


Fig. 2 : Graph of total albumin level decrease during the 10-day period ( $p < 0.005$ ).

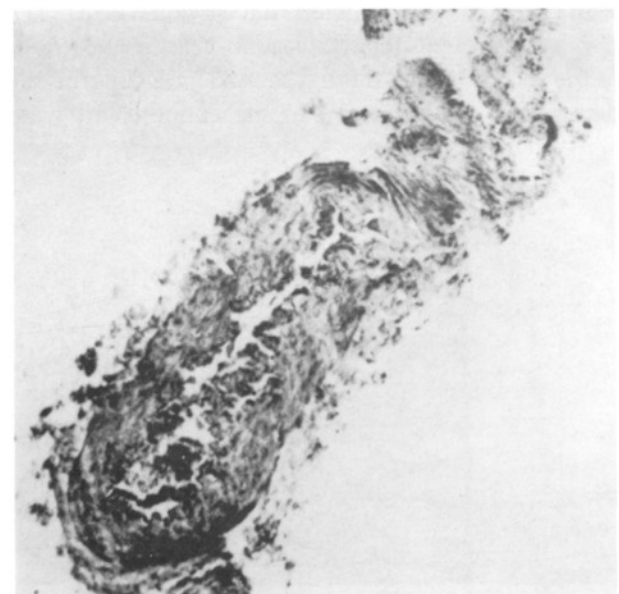
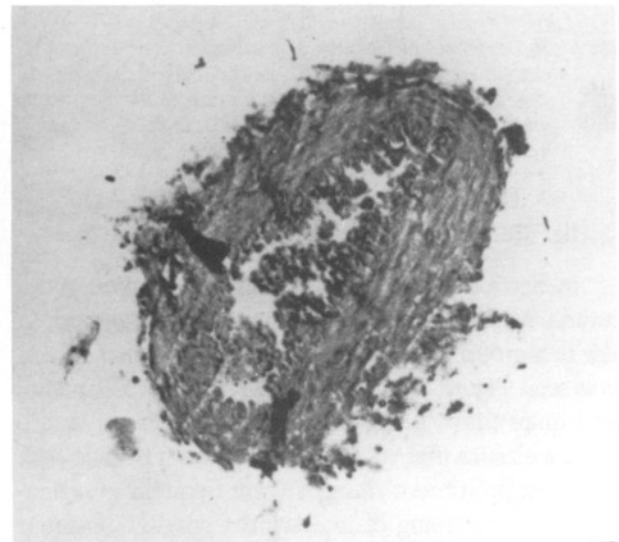


Fig. 3a, 3b

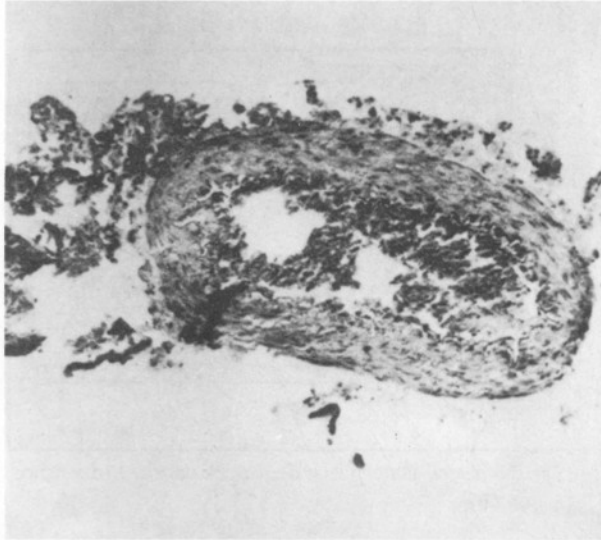


Fig. 3a, 3b, 3c : Photomicrographs of dog basilar artery in the first, second and control groups. Note the marked thickening of the medial layer and increase in internal elastic membrane, marked in the first group and moderate in the second group compared with the control group.

**iii. Electrone microscope changes:**

In electrone microscope studies degeneration of the intima, media and adventitia layers were observed. In the first group patchy detachment of endothelial cells was seen (Figure 4a). The basal membrane was lamellar and quite thick. Invaginations were observed in the lamina elastica interna. In medial smooth muscle cells the most prominent changes were frequent invaginations and widening of intercellular space, associated with increased collagen fibres. Mitochondrial degeneration was present in cytoplasmic cells (Figure 4b). In the second group, there was moderate endothelial detachment. Vacuolization in the endothelium was



Fig. : 4a

rarely seen. The invaginations in medial smooth muscle cells were less prominent than in the first group. The intercellular space was wider than that of the control group. In a small number of axons degeneration was seen in the adventitia (Figure 5,6a,6b).

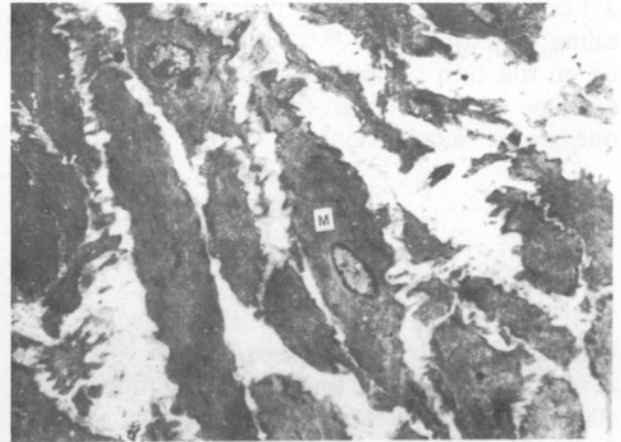


Fig. : 4b



Fig. : 5

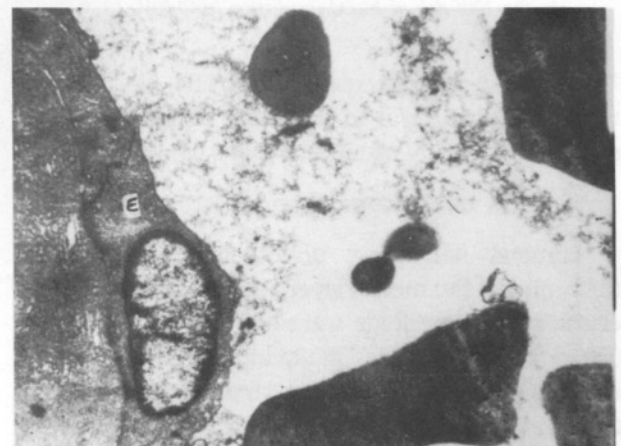


Fig. : 6a

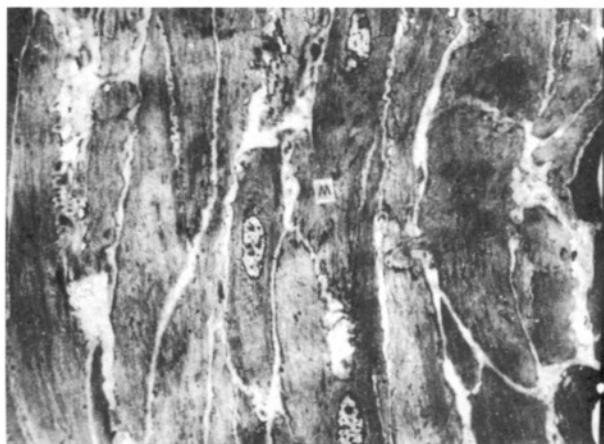


Fig. 4a, 4b, 5, 6a, 6b : Electronmicrographs showing the intima, media and adventitia of the dog basilar artery of the first and second groups compared with the control group (E=Endothelium B=Basal membrane, L=Lamina elastica interna, M=Media, Adventitia)

## DISCUSSION

Erythrocytes start to be destroyed in the acute phase after subarachnoid hemorrhage and in a few days oxyhaemoglobin increases and decreases with time and subsequently the bilirubin level increases (1). In vitro on days 0, 3 and 10 free fatty acids and bilirubin increase apparently while protein on the other hand decreases significantly in incubated blood (3). In our in vitro study incubated blood samples were also examined on days 0, 3 and 10 for protein and bilirubin levels. The bilirubin levels increased from  $0.20 \pm 0.03$  mg/dl on day 0 to  $7.75 \pm 0.73$  mg/dl on the day 10. Protein levels were found to be  $2.67 \pm 0.37$  g/dl on the 10th day while they were  $3.04 \pm 0.03$  on day 0. These findings conformed with the literature (3). The heme component of hemoglobin is very toxic for the subarachnoid space. In the days following subarachnoid hemorrhage due to decreased protein levels and increased free fatty acids bound to protein, the amount of bilirubin bound to protein decreases and bilirubin levels continue to be high and consequently become toxic. Bilirubin not bound to protein can penetrate protein and lipid membranes containing phosphatidyl choline, so destroyed phospholipids are transformed into polyunsaturated fatty acids. The enzymatic or non-enzymatic destruction of polyunsaturated fatty acids forms vasoconstrictor metabolites. This effect of bilirubin is not dose dependent, reaching a threshold level is enough (1 mg/dl) (1,3). As reported

in the literature, in light microscopic examination on the 10th day after application of erythrocytes, hemoglobin and bilirubin to the vessels, thickening of the vessel wall, destruction of the normal structure of the endothelium and elastic lamina and narrowing of the lumen is observed (4,7). After application of erythrocytes hemoglobin, and bilirubin to the vessels, electron microscopic studies show that there is endothelial cell swelling, vacuolization in the endothelium and smooth muscle cells, axon degeneration and varicosities in the adventitia. Our findings were similar (3,4,7). We also found mitochondrial and nuclear degeneration in the endothelial and smooth muscle cells. Especially in the first group there was widening of the intercellular space and degeneration of axon varicosities in the smooth muscle cells. In our study as bilirubin concentrations were constant in both groups, the prominent changes shown under the electron and light microscopes were due to plasma. In vitro studies have shown that the free fatty acid in plasma increases 10 fold on the 10th day (3). Most of these free fatty acids are arachidonic acid. Polyunsaturated fatty acids undergo peroxidation either by the non-enzymatic (free radical reaction) or the enzymatic pathway (arachnoid cascade). Hydroperoxides of polyunsaturated fatty acids are formed by non-enzymatic lipid peroxidation. 15-hydroperoxy arachidonic acid (15-HPAA), and 13-hydroxylinoleic acid (13-HPLA) produced at the end of arachidonic and linoleic acid peroxidation are found to have a vasoconstrictor effect on the basillary artery of the dog. In our study in the first group, blood given intracisternally had higher amounts of free fatty acids than reported in the literature (3). Due to destruction of arachidonic acid some metabolites are formed, the two important ones being 5-Hydroxyeicosotetraenoic acid (5-HETE) and 12-Hydroxyeicosotetraenoic acid (12-HETE) both of which are potent chemotactic factors for neutrophils. When these are found in the tissue the neutrophils move transcellularly from the endothelium to the vessel wall, damaging the endothelium in the meantime and causing formation of oxygen radicals. Endothelial damage causes platelet aggregation and so vasoconstriction like thromboxane A<sub>2</sub> and serotonin are secreted. Neutrophils on the other hand inhibit endothelial-based relaxation factor (EBRF) and prostaglandin 12 synthase (PS 12) which are secreted from endothelial cells and have a vasodilatory effect. 5-HETE and 12-HETE not only have this effect on neutrophils, they also have a vasoconstrictor effect

on the smooth muscle of vessels and cause organic changes (1). Fibrin degrading products and thrombin are most important amongst other known vasoconstrictors found in plasma. Fibrin degrading products have been found to have no important role in cerebral vasospasm (8), but thrombin plays an important role by enhancing the accumulation of thrombocytes in areas of damaged endothelium and by stimulation of serotonin secretion from these cells. Some unknown factors of plasma are thought to have an enhancing effect on vasospasm (7). As polyunsaturated fatty acids may play an important role in vasospasm, there is need for further and more detailed studies.

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