REVISTA ESPAÑOLA DE FISIOLOGIA
R. esp. Fisiol., 24, n.º 4, págs. 155-160, 1968

Institute for Cytology Research
Valencia (Spain)
(Dr. G. Forteza-Bover)
and
Department of Clinical Pathology
School of Medicine
Valencia (Spain)
(Prof. J. García-Conde)

# The Effect of an Synthetic Heparinoid (SP-54) on Platelet Aggregation

by

J. Aznar, R. Baguena, * J. García-Conde and V. Alberola

(Received for publication on April 22, 1968)

The heparin's anticoagulant property is well known, but some of its effects such as the lipolytic one (5) and inhibition of platelet aggregation (6, 8, 9, 11, 12) are less definite. It is posible that some compounds of similar structure possess more lipolytic and anti-aggregating activity and less anticoagulant effect than heparin, that could undoubtedly be useful in the clinic.

We have studied a sulphonic polysaccharide (SP-54) ** of heparin-like structure, which presents a marked fibrinolytic action (2, 4) and little anticoagulant effect. We report here our findings on the antiaggregating activity of this compound on platelets.

## Material and Methods

Citrated bovine blood was used (30 ml of 19 % sodium citrate per 1000 ml of blood). The citrated blood was then centrifuged at 400 g, for 20 minutes at 4° C in a MSE Mistral 4L centrifuge giving a supernatant containing about, 1.5 por $10^5$ platelets/cmm and few erythrocytes and leucocytes. Two units of heparin per ml of plasma were added to the platelet-rich plasma (PRP) to prevent clotting, dosis at which platelet dose aggregation is not impaired (1). Platelet poor plasma (PPP) was obtained buy centrifuging PRP at 2000 g for 30 minutes. Platelet aggregation was measured by a turbidimetric method (1, 3) and the results given in absolute and percentage figures. The heparinized PRP was incubated with the SP for une hour at room temperature (aproximately 20° C) and then measured platelet aggregation.

## Results

Twenty-five differents plasmas were studied, each incubated with different amounts of the SP. The average of 25 curves of each amount was then obtained. As can be seen from figure 1 as the dosis of the SP was increased the ability of platelets to aggregate decreased, i.e., as the OD decreases, the index of platelet

* Chairman Department of Clinical Pathology. School of Medicine. Santiago de Compostela (Spain).

** The SP-54 was suppliced by Lacer, S. A.

| mg/ml | OPTICAL DENSITIES | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 0.00 | 70 | 46 | 40 | 39 | 39 | 41 | 43 | 46 | 48 | 49 | 51 |
| 0.006 | 71 | 46 | 41 | 39 | 40 | 42 | 45 | 47 | 50 | 54 | 57 |
| 0.12 | 70 | 62 | 60 | 61 | 63 | 65 | 67 | 70 | 73 | 76 | 79 |
| 1.50 | 71 | 65 | 64 | 65 | 66 | 67 | 69 | 72 | 75 | 77 | 80 |
| Tie. Mi. | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |

| mg/ml | A.I. |
|---|---|
| 0.00 | 53 |
| 0.006 | 55 |
| 0.12 | 83 |
| 1.5 | 91 |

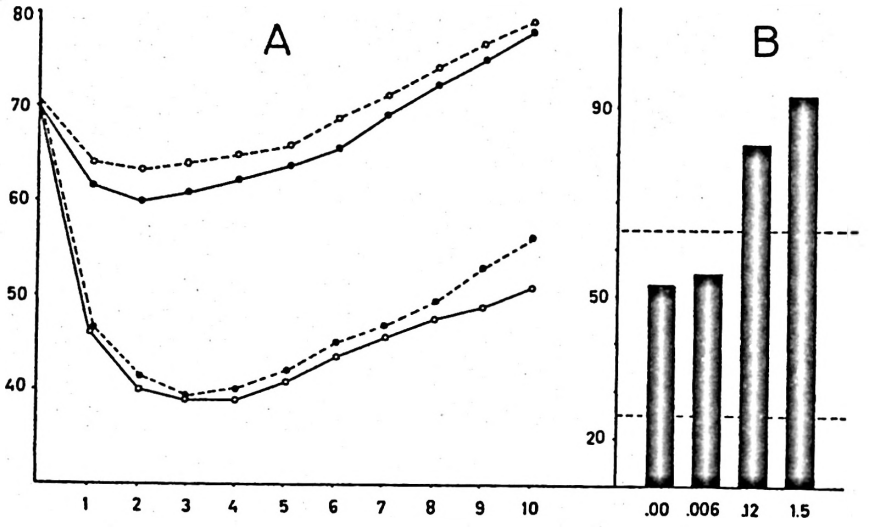



FIG. 1. Platelet aggregation curves obtained by using different dosages of sulphonated polysaccharide. A. Above. Optical densities obtained with each dosage used. Below. Graphic representation of the curves. Ordinates: Optical density. Abcissas: time in minutes. B. Above. Aggregation index obtained with each of the dosages. Below. Ordinates: aggregation index. Abcissas: dosage of the drug used. The broken lines indicate the normal limites.

aggregation increased (the higher the index, the lower ability of thrombocytes to aggregate). These increments were in close relation with the dosis of the SP used. It can be seen that with 0.12 mg/ml, of plasma the index is 83, i.e., outside the limits mentioned above.

Since the increase in OD could be caused by some collateral effect of the compound on the plasma and not be in the least in relation to the platelet aggregation, the sulphonated polyanion was incubated in the same manner as in the previous group, but using plasma without platelets. In a first set of trials, in which no sulphonated polysaccharide was used, we confirmed (fig. 2-B, first column) that there was a progressive increase in the OD. This was undoubtedly due to the formation of insoluble calcium citrate since the excess sodium citrate reacts with $CaCl_2$, which had been added at the beginning of the increases then paralleled those obtained in the trials without the drug (fig. 2-A). It does not then seem that once agitation began the sulphonated polysaccharide caused any increases in the OD of a plasma without platelets.

If the OD increase obtained in the 25 PRP incubated with the drug was not due to the polysaccharide's action on the plasma it could be due to some other action on the platelets, independent of its aggregating function, for example, it may pro-

TABLE I

Quantitative study of the anti-aggregating activity of the SP (0.12 mg/ml)

| A | B | C | D | E | F |
|---|---|---|---|---|---|
| 1 | 4.3 | 1.7 | 2.6 | 16 | 13.4 |
| 2 | 6.4 | 2.6 | 3.8 | 20 | 16.2 |
| 3 | 7.6 | 3.6 | 4.0 | 22 | 18.0 |
| 4 | 10.0 | 4.3 | 5.7 | 24 | 18.3 |
| 5 | 11.8 | 5.6 | 6.2 | 24 | 17.8 |
| 6 | 13.7 | 6.4 | 7.3 | 24 | 16.7 |
| 7 | 15.3 | 8.6 | 6.7 | 24 | 17.3 |
| 8 | 16.6 | 9.5 | 7.3 | 24 | 16.7 |
| 9 | 18.7 | 10.1 | 8.6 | 25 | 16.4 |
| 10 | 21.0 | 11.1 | 9.9 | 27 | 17.1 |

A. Time in minutes.

B. Increases of the OD from the beginning of the trials to the minute being considered. These values were obtained by subtracting, in the figure 3B, the OD at minute zero from the corresponding minute.

C. Increases of the OD due to the formation of calcium citrate.

D. Difference between B and C. Real increment of OD due to the SP.

E. Increases of the OD due to the aggregation that was produced when the sulphonated polysaccharide was added to PRP.

F. The values were obtained by subtracting column D from E. They correspond to the real increase of the OD caused by the drug's action on platelet-rich plasmas. This reflects its anti-aggregating platelet activity.

duce their lysis. In order to confirm this last point the drug was incubated (at the same concentration as in the first trial) with a PRP but without adding $CaCl_2$. There was not platelet aggregation. The concentration, agitation and the rest of the variants were identical to those of the first group. Twenty five different plasmas were studied by this method. It was observed (table II, columns B, C, E, D) that the sulphonated polyanion hardly produced any increase in the OD when it acted on non-aggregating platelets, not even at high dosages.

TABLE II

Study of the action that sulphonated polysaccharide has on PRP

| A | B | C | D | E | F | G | H | I | J |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 145 | 149 | 148 | 149 | 3 | 2.6 | 0.4 | 13.4 | 13 |
| 2 | 144 | 149 | 149 | 150 | 5 | 3.8 | 1.2 | 16.2 | 15 |
| 3 | 144 | 149 | 150 | 150 | 6 | 4.0 | 2.0 | 18.0 | 16 |
| 4 | 143 | 148 | 150 | 150 | 7 | 5.7 | 1.3 | 18.3 | 17 |
| 5 | 143 | 148 | 151 | 150 | 8 | 6.2 | 0.7 | 16.7 | 16 |
| 6 | 142 | 147 | 151 | 151 | 9 | 7.3 | 1.7 | 16.7 | 15 |
| 7 | 142 | 147 | 151 | 151 | 9 | 6.7 | 2.3 | 17.3 | 15 |
| 8 | 142 | 146 | 152 | 151 | 10 | 7.3 | 2.7 | 16.7 | 14 |
| 9 | 142 | 146 | 152 | 151 | 10 | 8.6 | 1.4 | 18.4 | 17 |
| 10 | 142 | 146 | 152 | 152 | 11 | 9.9 | 0.1 | 17.1 | 17 |

A. Time in minutes.

B. Aggregation curve with PRP but without the addition of $CaCl_2$ nor SP.

C. The same but with 0.006 mg of SP per ml of plasma.

D. The same but with 0.12 mg/ml.

E. The same but with 1.5 mg/ml.

F. Differences at each minute between the optical densities of columns D and B.

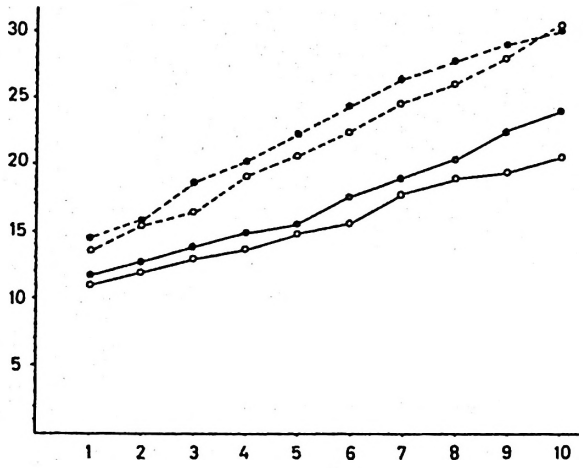
G. Increase of the OD caused by the drug acting on plasma without platelets (taken from the table I column D).

H. Differences between the optical densities of F and G.

I. Increase of the OD at the minutes being studied, caused by the anti-aggregating action of the SP (taken from table I column F.

J. Real increase of the OD caused by the drug's action on platelet-rich plasma. This increase in OD reflects the anti-aggregating platelet activity of the SP.

A




B

| T. M. | OPTICAL DEN. | | | |
|---|---|---|---|---|
| 0 | 9.2 | 9.3 | 9.0 | 10.7 |
| 1 | 10.9 | 11.6 | 13.3 | 14.4 |
| 2 | 11.8 | 12.4 | 15.4 | 15.6 |
| 3 | 12.8 | 13.6 | 16.6 | 18.4 |
| 4 | 13.5 | 14.7 | 19.0 | 20.3 |
| 5 | 14.8 | 15.3 | 20.8 | 22.8 |
| 6 | 15.6 | 17.6 | 22.7 | 24.2 |
| 7 | 17.8 | 18.7 | 24.3 | 26.0 |
| 8 | 18.7 | 20.4 | 25.7 | 27.7 |
| 9 | 19.3 | 22.2 | 27.7 | 28.7 |
| 10 | 20.3 | 24.3 | 30.0 | 29.9 |
| mg/ml | 0.00 | .006 | 0.12 | 1.5 |

FIG. 2. Action of sulphonated polysaccharide on a plasma without platelets. A. Ordinates: optical densities. Abcissas: times in minutes that each experience lasted. B. Optical denstities obtained at each trial. The curves that are shown here are the median of the 25 trials that were carried out with different dosages of the drug.

## Discussion

That the increase in OD obtained when PRP was incubated with the sulphonated polyanion was due to its action neither on the plasma nor on the platelet stroms, it suggest that its effect might be preventing the thrombocytic aggregation. We have seen above how when plasma without platelets was recalcified its OD increase, as it will be indicate below, it could not be responsible for the increase in OD which was observed in the first group of experiences in which the sulphonic polyanion acted on platelets capable of aggregation. On the other hand, in the trials in which recalcified PPP and the SP were used, the OD increase obtained, was slightly higher than when polysaccharide was not used (fig. 2). This indicated that even without platelets the drug produces a slight increase obtained in the first group of experiences (fig. 1) was not due to a decrease in platelet aggregation but to the drug's action on the plasma. However, on making a more detailed quantitative study, it was observed (table I) that when plasma without platelets was incubated with 0.2 mg of the drug per ml of plasma, these OD increases were smaller than those obtained when PRP was used under the same experimental conditions.

We can see that table I, column A represents the time in minutes that each trial lasted and column B the increases in OD from the beginning to the minute being considered. These values were obtained by subtracting on fig. 2-B, the OD at minute zero from the corresponding minute; in the experiment using 0.12 mg of problem per ml of plasma, for example, in column B or the table I, the 4.3 of the first minute, was obtained by subtracting 9.0, the reading at the first minute, from 13.3 which was the initial reading of the curve obtained by using 0.12 mg of the SP per ml of plasma (fig. 2-B). Column C represents the increase in OD due

to the formation of calcium citrate between the minute zero and the minute being considered. The datum has been taken from the first column of fig. 2-B. By subtracting 9.2 from 10.9, the value of 1.7 corresponding to the first minute on column C of table I, was obtained. If the increase in OD caused by the formation of calcium citrate (column C) is subtracted from the total OD (column B) the difference will be that due to the action of the drug on the plasma (column D). We have already seen how the increase in OD obtained when the drug was incubated with a PRP, is higher (fig. 1). By calculating the OD difference existing at each minute betwen the trial that was done using PRP with the SP and that without it (fig. 1) we obtained the data, column E, of the table I. This increase should be due to the anti-aggregating effect of the drug, but we have previously seen that the drug caused an increase in the OD of a plasma having no platelets and so it cannot be caused by its anti-aggregating action (column D, table I). If we subtract this increase from the total global increase (subtract column D from E) we will obtain the real increase in OD caused by the anti-aggregating action of the problem solution (column F). For example on the first minute (column F, table I) there is an increase of 13.4 in the OD between the trials with and without the problem solution. We have already shown that this OD increase can only be due to the anti-aggregating action of the drug. Therefore, the OD increase when incubating a PRP with a SP was not due to the action on the plasma itself.

We shall now discuss if it could be due to its effect on non-aggregating platelets and so be independent of the platelet function. We have previously demonstrated that even though the drug produce a slight OD increase when incubated with a plasma rich in non-aggregating platelets (table II, C, D, E). A quantitative study of the increase showed that they were always inferior to those obtained with a plasma rich in aggregating platelets. Table II, columns B, C, D and E shows the median curves obtained after experimenting with 25 different PRP. Calcium chloride had not been added an so no aggregation was produced. Column B shows the values of the curve obtained without the addition of the drug. Columns C, D and E have the values obtained by using 0.006 0.12 and 1.5 mg of problem per ml of plasma respectively.

The OD increases were very small. Column F indicates the differences between the OD of column B (without polyanion) and column D (with 0.12 mg per ml) at the minute being considered. This number would represent the increase in OD caused by the drug on platelet that do not aggregate. We must, however, subtract the increase caused on a plasma without platelets, column G. Therefore, the difference between both ODs would represent the real increase in OD caused by the drug on a plasma rich in non-aggregating platelets (column H). Column I represents the increases caused by the same dosage of the drug on aggregating platelets (from table I, column F). The values in this column were always highes than those in column H. Since the OD increases were obtained neither with non-aggregating platelets nor with plasma without platelets, it demonstrates that they were not due to plasma modification nor to alterations of the platelet structure.

It can be deduced that the sulphonated polysaccharide studied acted as a platelet anti-aggregating substance.

As for its form of action, we believe that this substance might inhibit platelet aggregation through the liberation of peptides from the fibrinogen and that might be adsorbed to the platelet membrane altering its ability to aggregate. This effect could also result from lipolytic activity of the compound modifying the lipoproteins

and phospholipids of the platelet membrane, since it has been shown (7) that the platelet membrane plays a principal role in its aggregating function.

ACKNOWLEDGEMENTS. The authors are grateful for the tecnical assistance of Rosa María Ruiz.

## Summary

The antithrombotic activity of a synthetic heparinoid, SP-54, has been studied. It was demonstrated that the SP-54 at therapeutical doses, prevents *in vitro* platelets from adhering to each other. It could suggest the use of this drug to prevent the thrombus formation in the thrombophilic state.

## References

1. AZNAR, J.: *Hemostase*, **6**, 351, 1966.
2. AZNAR, J., and LASSO, M.: *R. Clin. Esp.*, **91**, 283, 1963.
3. AZNAR, J., LASSO, M., and FERNÁNDEZ PLA, A.: *Nature*, **213**, 1251, 1967.
4. COCCHERI, S., GENARI, P., and LORETTI, A.: *Clin. Terap.*, **25**, 3, 1963.
5. HAHN, P. F.: *Science*, **98**, 19, 1943.
6. HELLEM, J. A.: *Scand. J. Clin. Lab. Invest.*, **12**, suppl. 51, 1960.
7. MARCUS, A. J., D. KUCKER-FRANKLIN, L. B.: *J. Clin. Invest.*, **45**, 14, 1966.
8. MUSTARD, J. F., and MURPHY, E. A.: *Blood*, **22**, 1, 1963.
9. NORDOY, A., and ODEGARD, E. A.: *Scand. J. Clin. Lab. Invest.*, **15**, 399, 1963.
10. PARAMELLE, B.: *Therapy*, **17**, 719, 1962.
11. ROBERTS, B., ROSATO, F. E., and ROSATO, F. E.: *Surgery*, **55**, 803, 1964.
12. ZUCKER, M. B.: *Amer. J. Physiol.*, **148**. 275, 1947.