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QUANTIFICATION OF DRUG-STIMULATED COLLAGEN FIBRILLOGENESIS WITH A GRANULOMETRY ALGORITHM BASED ON MATHEMATICAL MORPHOLOGY

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ABSTRACT

Individual tropocollagen molecules of acid soluble type I collagen present a spontaneous tendency to self-aggregation. Many molecules aggregate in an ordered way to form fibrils and fibers. In "in vitro" conditions this fibrillogenesis takes place rather slowly and only to a limited extent. It was demonstrated in this work that some flavonoids and bioflavonoids are able to accelerate considerably the collagen fibril formation and in presence of some of the substances tested the size of the formed fibers is much greater than that of the fibers formed by spontaneous self-aggregation.

In such a work the exact determination of the size and the shape of the aggregates is of utmost importance. This evaluation was done with a granulometry algorithm based on mathematical morphology. The size of the particles is obtained by successive openings on a binary image obtained on thresholding or with the top-hat transformation of a digitized image. The total original surface of the particles and the remaining ones after each opening are given as well as the individual surface of each particle after labeling. The results given in this paper and some others which will be published separately, demonstrated the power of this granulometry algorithm and its usefulness for biomedical applications.

Keywords: collagen type I, fibrillogenesis, granulometry method, quantification.

INTRODUCTION

Interstitial collagens (mainly type I and also types II and III) are quantitatively the most important components of many tissues where their functional rôle is also considerable. Such is the case for instance of the vascular walls, which loose a great part of this collagen simply with age and even more in pathological conditions (Svejcar et al., 1963; Bouissou et al., 1976). It was shown for instance that varicous veins loose up to 60% of their collagen (Niebes, 1987). The smooth muscle cells of the vascular wall are able to synthetize collagen, but this biosynthesis is not sufficient by itself, the individual tropocollagen molecules have to aggregate in definite patterns in order to form functionally valid crosslinked fibrils and fibers (Denèfle et al., 1987). The mechanism of

this fibrillogenesis is not yet perfectly understood, the importance of the propeptides of procollagen in this phenomenon was proposed (Fleischmajer et al., 1990).

Acid-soluble collagen molecules have self-aggregating properties, but the spontaneous formation of fibrils is slow and takes place only to a limited extent (Bouligand et al., 1985). It was noticed in pharmacological studies that some drugs are able to stimulate collagen fibrillogenesis. Such an effect is of great importance for the explanation of the mechanism of action of such drugs. Therefore we looked for a method able to evaluate quantitatively the ability of a given drug to stimulate collagen fibrillogenesis. It was noticed that the size and shape of the collagen particles varies according to the drug tested and to the experimental conditions, so the most appropriate evaluation of the effect appeared to be the study of these properties of the collagen particles by granulometry. A new software written in Fortran 77 was worked out for this purpose and it was tested on native collagen and drug-stimulated collagen gels. This granulometry algorithm was implemented on a micro-computer working with a Morpholog software (Noésis, France) based on the transformations of the mathematical morphology (Serra, 1982). We shall here describe the granulometry procedure and the results we obtained with it.

MATERIALS AND METHODS

1. Preparation of collagen gels for granulometry

0,5 ml of acid-soluble type I collagen solution at 0,5% in acetic acid 0,5M was added to a Petri dish with a diameter of three and a half centimetres. Gelling takes place immediately after the collagen, incubated beforehand with a flavonoid or a ß-lactam during three hours at 37° C , and the 1% melted agarose in a 0,1M TRIS-HCl buffer at pH 7,5, (1:1 v/v), have been mixed. The flavonoids (Diosmin, RDEG, a non commercial preparation, Ginkgo, a procyanidolic oligomer (PCO)) and the ß-lactam, were used so that their final concentration in the gel was 0,1 mg/ml. The control sample was incubated in the same conditions with the same amount of buffer. The agarose was used as a support for collagen.

Furthermore the collagen gels were stained with Sirius Red 0,1 % in saturated picric acid to visualize the fibrils in the gel.

2. Analysis of the gels

The stained collagen gels, those incubated with a flavonoid or ß-lactam as well as controls, were examined under an Olympus microscope with 4x magnification. In this condition 1 pixel = 7,99 µ in length and represents a surface of 63,995µ2. A Vidicon-camera (Sofretec, France) is fitted to the microscope and the obtained images are digitized and stored in a MVM 600 analog-digital/digital-analog converter (Sofretec, France, 512 x 512 x 8 bits). Image analysis is performed using a BFM 186 micro computer (Inter Instruments, 768 ko). Images are visualized on a M31 (Sofretec) display console.

Once the image digitized into 256 x 256 pixels, each of them received a grey level value (between 0 and 255) proportional to the amplitude of the video signal. The parts of the image containing the darkest pixels which correspond to the collagen fibrils were extracted by the "top hat" algorithm (Meyer, 1978). In some cases it is possible to extract the particles to be studied by simple thresholding. The binary image obtained by one of these operations is formed of pixels with the value 1 for those corresponding to

collagen fibrils and the value 0 for all the others. The granulometry algorithm was applied to these binary images.

The obtained results were submitted to the usual statistical analysis which included for each series of measurements the calculation of the means, the standard errors etc. The means of two different series of measurements were compared with the Student t-test in order to estimate the significance of their difference.

RESULTS

1. The granulometry algorithm

Granulometry analyses the size-distribution of particles of any kind. The name comes from the method of sieves with decreasing mesh-sizes which were originally used to separate solid particles (grains) of different sizes. The first sieve retains the greatest particles and soforth while the smallest particles may pass trough the smallest sieve. Granulometry based on image-analysis, which has been introduced by G. Matheron (1967) follows the same principle.

For the analysis of the shape and the size of the collagen fibrils we have used a method based on the mathematical morphology (Serra, 1982). This method initially used to describe sand grains (Frossard, 1978) is based on iterative image transformations with a set of structuring elements (Raphael et al., 1985).

For the present study, we have used a set of hexagonal structuring elements with a diameter increasing from 3 to 21 pixels. Each opening results in the elimination of the original image of small parts of any particle which cannot contain the structuring element considered. The area of all the particles is measured at the beginning, and then after each opening we obtain the eliminated and the remaining area. This procedure gives us several informations on the morphological structure of the studied fibrils.

If x is the phase detected by the "top hat" algorithm, on thresholding after the opening by the nth structuring element we obtain:

$$X n = (X - Bn) + Bn \qquad (1)$$

where the diameter of Bn is 2 (n + 1) - 1 pixels.

For the edge-correction we have used the fraction of the surface area of X ($A_A(X)$) (Coster et al. 1989).

The relative variation of the fraction of the surface area is:

$$fn = (A_A(x) - A_A(x_{Bn})) / A_A (x) \qquad (2)$$

This is the distribution function, which is determined by the algorithm after each opening and is represented in a histogram at the end of the analysis. The number of hexagonal openings n required to eliminate all pixels gives us an indication of the size of the largest fibers. The variations of the size and shape of the distribution function histogram reflects the size and shape of the analyzed particles as follows:

1) The height of the first bar of the histogram is related a.) to the size and b.) to the shape of the analysed particles:

a.) a high first bar means numerous small particles

b.) a high first bar also suggests elongated and thin or irregularly shaped particles; on the contrary a small first bar is in favour of round and larger or regularly shaped particles

2) The profile of the histogram is also related to the shape of the particles: if the evolution of the histogram is rapid, the difference in the size of the successive bars is important, then the shape of the particles is likely to be irregular. In the opposite case a regular shape can be assigned to the analysed particles.

3) The number of the bars of the histogram is related to the size of the largest particles: the more bars compose the histogram, the greater is the size of the largest particles.

The granulometry software also integrated the possibility of "labeling", so individual sizes for each particle can be obtained on the original image as well as after each "opening" iteration.

So the granulometry software gives us for each microscopic field for the original image and after each opening the following informations:

1. the number of particles per field

2. the total surface of the particles

3. the average surface of a particle (the total surface divided by the number of the particles)

4. the histogram of the distribution function

5. the individual surface of each particle

6. the size distribution of the particles

All these informations were collected on 10 microscopic fields for the control native collagen gels and for those of each drug tested. 2 Petri dishes for each type of gel were analysed, 5 randomly distributed fields were studied on each of them.

2. The results obtained on native and drug-stimulated collagen gels

The main goal of this paper is the description of the granulometry software and not the discussion of the biological results. Therefore we will discuss here only the stereological parameters. The results obtained for the 3 first of them are given in Table I. All the figures are the average of 10 measurements, each of them on a different field. The results shown in this table were obtained with the concentration of 0,1 mg/ml of each tested substance.

2.1. The number of particles per field

One can see that the first parameter, the average number of particles per microscopic field increases progressively from the control gel (91,5) to that incubated with the PCO (176,8), the difference being 95 % of the control value (Fig.1.). However the greatest average number of particles per field was obtained with the substance extracted from the

Ginkgo tree: 218,5 particles per field. In this particular case the difference represents 141 % of the control value.

Table I. The first 3 stereological parameters obtained by the granulometry software for the different types of gels. The figures of the first 3 lines: number of particles per field, total area of the particles per field and the average area of a particle in a) are given in pixels. The average area of a particle in b) is given in μ2-s. All the tested drugs increase the number and the size of the collagen particles but not to the same extent.

Parameter	Control	ß-lactam	Diosmil	RDEG	Ginkgo	PCO
n particles / field	91,5±24,0	184,2±21,0	146,4±9,8	170,9±17,8	218,5±9,2	176,8±12,2
total area / field	1983,5 ±558,9	4931,9 ±620,8	4176,8 ±492,5	5162,7 ±600,4	7089,3 ±308,0	10829,3 ±1040,6
average area a)	21,9	26,8	28,6	30,2	32,4	61,3
of a particle b)	1401,5	1715,0	1830,3	1932,6	2073,4	3922,9

2.2. *The total surface of the particles*

The second parameter represented on Table I. and Fig.2., is the average total area(surface density) of the particles per field. One can see that all the tested compounds increased the average total surface of the collagen particles.

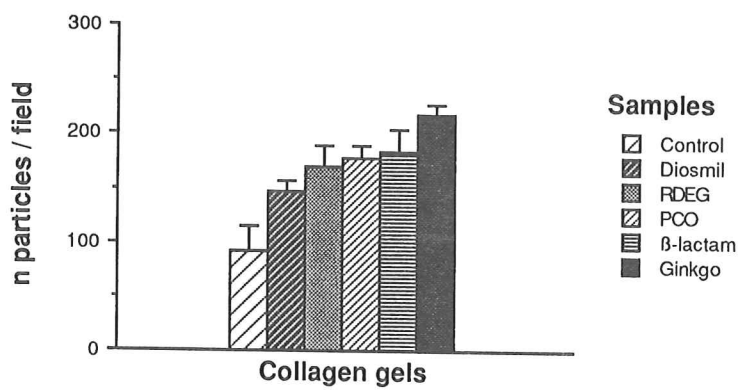

Fig.1. The average number of particles per microscopic field increases compared to the control with all the tested substances. For this parameter Diosmil was the less effective, Ginkgo the most effective.

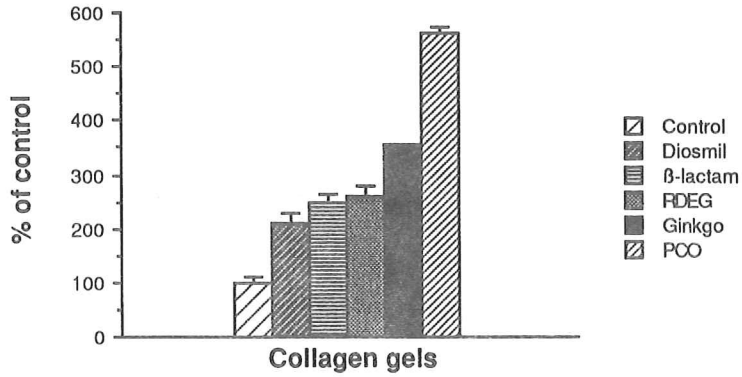

Fig.2. Average total area of the collagen particles in the different gels.All the tested substances increase this parameter. Diosmil gave the smallest effect (2,1 times the control) but here the most intense effect was obtained with PCO: 5,46 times the control value. The intensity of the effect increases from the Diosmil (2,1 times the control) to the most effective substance (PCO) giving an average total surface density more than five times greater than the control. These differences to the control values are statistically significant: $p<0,005$ for Diosmil, ß-lactam and RDEG, while for Ginkgo and PCO the security was even higher: $p<0,0005$.

2.3. The average surface of a particle

The third parameter on Table I is the average area of a particle. One can observe a steady increase from the control value towards the PCO-incubated sample (Fig.3.). Compared to control, this parameter was increased by 22 % in samples treated with ß-lactam, by 30,5% with Diosmil, by 38% with the RDEG and by 48% with the Ginkgo extract. Still the most important increase in % of the control value was the average of the samples treated with PCO: +180% in comparison to the controls.

2.4. The histogram of the distribution function

For each analysis we obtain a distribution function histogram. The height of the bars and the profiles of the histograms obtained for ex. for a control experiment and an other with the PCO give the following types of informations:

The first bar of the control histogram is rather small, this is an indication for round or regularly shaped particles; there are only two bars, this means that the size of all the particles present is rather small. It also shows that the total surface eliminated by the second opening is greater (824 pixels) than that eliminated by the first opening (521 pixels).

The histogram obtained with the PCO has 4 bars, this means that there are much greater particles present than in the control experiment. The small first bar and the profile of the histogram are in favour of regularly shaped particles. The height of the bars show that

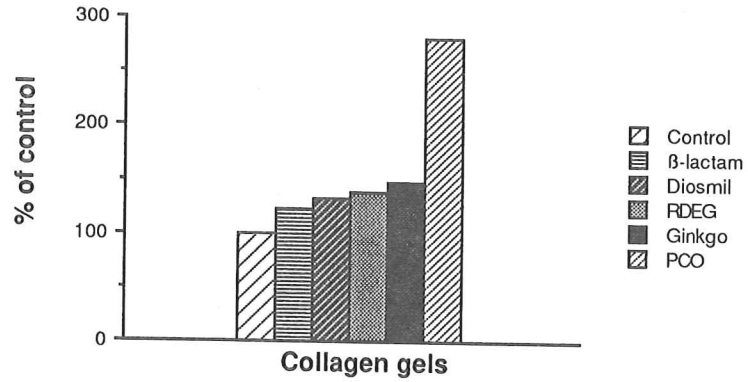

Fig.3. Average area of a particle in each of the gels. Compared to the control, all the studied compounds increased the average particle size. The greatest increase, +180%, was obtained with the PCO, the smallest, +22%, with the Diosmil incubated collagen.

the second opening eliminates the greatest total surface (4541 pixels), and that the total surface of the particles eliminated by the first opening (1747 pixels) is only 17% of the initial surface (10216 pixels), against 35% for the same value in the control experiment: 521 pixels eliminated out of an initial surface of 1478 pixels. So the histograms give indications on the size and the shape of the particles as well as on the relative proportion of the particles of different sizes.

2.5. The individual surface of each particle

The Morpholog (mathematical morphology) software gives the possibility to label individually each particle present on a binary image, and on a label-image several stereological parameters can be obtained separately for each particle present on the image. This possibility was integrated in our granulometry method, so we can collect the size of each particle separately. In an exemple of such a listing : 180 particle-sizes are listed for the presented microscopic field. The smallest particles have a surface of only 9 pixels (9x64 µ2), (n°-s 81, 94 and 170), the greatest is of 776 pixels (766x64 µ2), (n°72). It can be noticed that the small sized particles predominate in the given example.

2.6. The size distribution of the particles

As we can obtain the size of all the particles, we have the possibility to study their distribution in several size-classes. For the delimitation of the size-classes we may have logical reasons, for instance when we study cells of different sizes such as red blood cells and different white blood cells. In other cases the sizes must be arbitrarily delimited.

We distributed the native and drug-stimulated collagen particles in four different classes of increasing size (Fig.4.) One can see that the native controls have most of their particles (56%) in the first class which contains the smallest particles (11 - 20 pixels = 704 - 1280 µ2), 23% and 20% in the second (21 - 30 pixels = 1344 - 1920 µ2) and third (31 -

100 pixels = 1984 - 6400 μ2) classes and only 1% in the fourth class (101 - 1000 pixels = 6464 - 64000 μ2).

With the drug-stimulated samples the proportion of the smallest particles decreases and that of the greater particles increases in function of the stimulating capacity of the tested substances. With the most efficient stimulator (PCO) the first class contains only 37% of the particles, approximately the half of the remaining ones is in the second (19%) and third class (33%), and the fourth (101 - 1000 pixels) contains 11% of the particles.

In all cases about half of the particles fits in the second and third class, what means that their size is between 21 and 100 pixels. When we try to establish the hierarchy of the stimulating efficiency, we find progressively increasing size-values in the third class from the controls to the PCO-stimulated gels. But in the other classes this progressivity is less evident (1st and 4th classes) or even absent (2nd class).

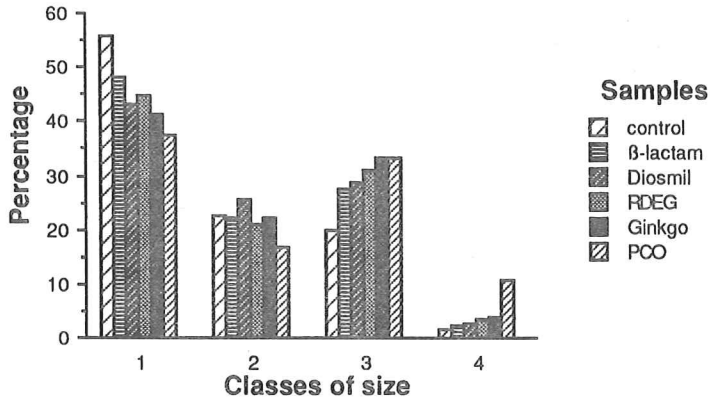

Fig.4. The first class contains the particles from 10 to 20 pixels, the second from 21 to30, the third from 31 to100 and the fourth from 101 to 1000. The control gels have more than 50% of their particles in the first class and only 1% in the fourth. With the stimulated gels the proportion of the smallest particles decreases and that of the greater ones increases. The PCO stimulated gels produce the greatest particles.

DISCUSSION

The granulometry algorithm described in this paper is based on mathematical morphology and works by successive openings with a structuring element of progressively increasing size. The data obtained are the initial surface of the particles and after each opening the remaining and the lost surfaces, all in pixels. The distribution function is also calculated after each opening, and a histogram of this function is drawn at the end of each analysis. With the label function one can obtain the individual surfaces of each particle present in the analysed field.

This granulometry algorithm was applied to the study of spontaneous and drug-stimulated collagen fibrillogenesis. We could demonstrate that some flavonoids or bioflavonoids are able to stimulate to divers extents the formation of collagen fibers. The most potent effect was obtained with the procyanidolic oligomers (PCO), which provoked the formation of the largest particles. Their intense action can be seen on the total surface of

the particles (Fig.2.), on the average size of a particle (Fig.3.). On these parameters the effect of PCO is more important than that of the other tested substances.

With the evaluation of the stimulating effect on collagen fibrillogenesis of five different substances this granulometry algorithm proved its usefulness as a tool in the evaluation of drugs acting on the fibrillogenesis of collagen.

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