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Milos Chvapil and Hana Holubec

Department of Surgery,
University of Arizona Health Sciences Center

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TEL. 東京 (664) 7750 (代表)

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Milos Chvapil and Hana Holubec

Department of Surgery,
University of Arizona Health Sciences Center
Tucson, AZ 85724, U.S.A.

ABSTRACT

The effectiveness of two types of collagen materials available as topical hemostatic agent, collagen fleece (Novacol) and fibrous form (Avitene), was ascertained by studying the magnitude of ^{14}C -serotonin release from human platelets, prelabelled with this amine.

It was found that collagen fleece fibers added to platelets in plasma promoted significantly higher release of ^{14}C -serotonin than an equivalent dose of fibrous form. The reasons for this effect are discussed in view of several reports that both hemostatic agents are equally effective in clinical (patient use) as well as in controlled animal tests.

INTRODUCTION

The role of platelets in thrombosis has been known for a long time¹⁾. Much later it was recognized that it is collagen, either of the basement membranes²⁾ or in a fibrillar form, which induces the sequence of adhesion-release-aggregation of platelets resulting in the formation of so-called white thrombus³⁾. Platelet interaction with collagen was utilized commercially in the development of various types of collagen-based topical hemostatic agents, currently used in large scale in various surgical disciplines.

Development of any collagen-based hemostatic material requires the evaluation of actual hemostatic effectiveness in both *in vitro* (platelets) as well as *in vivo* (animal) models. Using platelet-rich plasma, the addition of fine fibrillar collagen (such as the microfibrillar collagen,

fibrous form, with fibers 100 Å in length), which uniformly disperses in plasma changes the turbidity of plasma due to formation of larger aggregates with platelets⁴⁾. Turbidity is measured spectrophotometrically at 540 nm. However, such a test is impossible to use if collagen fibers exist in a longer form, as in the case of collagen hemostatic fleece. Uneven distribution of long collagen fibers in the plasma makes the readings of the turbidity an unreliable method to ascertain the magnitude of platelet aggregation by collagen. In this case, another method of determining the function of platelets under the effect of fibrillar collagen must be used. Once platelets adhere to collagen, they release substances, such as ADP and serotonin⁵⁾, which enhance further aggregation of platelets to form a clot. Close correlation was found between magnitude of platelet adhesion and serotonin release induced by collagen. Detection of serotonin from ¹⁴C-serotonin prelabelled platelets has been routinely used to assess the effectiveness of a certain type of collagen to affect the various functions of platelets⁶⁾.

This study compares two commercial collagen hemostatic agents existing in a fibrillar form in their effectiveness to activate platelets. The magnitude of platelet activation was measured by their release of serotonin.

MATERIALS AND METHODS

The Hemostatic Collagen Fleece (collagen fleece, Novacol, Bioplex Corp., a subsidiary of Datascope Corp., NJ, U.S.A.) used in this study was isolated from bovine achilles tendons by a combined chemical and mechanical patented procedure⁷⁾. Microfibrillar Collagen Hemostat (fibrous form, Avitene) was supplied by Avicon Incorporated, Fort Worth, TX. This product was manufactured by a procedure described by Battista and Cruz⁸⁾. While the collagen fleece nonwoven matrix consists of fibers up to 4-5 cm long with an average length of 2.0 cm, fibrous form fibers have a more uniform distribution, averaging 100 Å in length. Thus, the latter product forms a loose powder.

The method of measuring ¹⁴C-serotonin release from collagen activated platelets consists of three steps:

1 Preparation of platelet-rich plasma

One hundred milliliters of human blood was mixed 1:4 with 3.8% citrate, centrifuged at 1400 rpm for ten minutes in nalgene tubes to obtain platelet-rich plasma (PRP) supernate. After removing PRP the sample was recentrifuged at 2400 rpm for ten minutes and platelet poor plasma (PPP) was obtained.

2 Prelabelling of platelets with ¹⁴C-serotonin

To label the isolated platelets with ¹⁴C-serotonin (5-hydroxytryptamine-3-creatine sulfate-¹⁴C, 50 µCi/µmole, Amersham-Searle IL, USA), 2.2×10^7 cpm of the marker in 30 µl volume was added to 10 ml PRP, incubated for 15 minutes and centrifuged. The supernate was removed, pelleted platelets resuspended and washed several times in Hepes buffer enriched in

Table 1 Effect of collagen hemostats on the ^{14}C -serotonin release by human platelets

Sample ¹⁾	^{14}C -Serotonin ²⁾ Release	Significance ³⁾	In % of Total Activity
Control (no fibers)	1045 \pm 7	a	4
Collagen fleece	5240 \pm 33	c	22
Fibrous form	1825 \pm 17	b	8

¹⁾ 10 mg of fibers were added to 2 ml of PPP and 8 ml of ^{14}C -serotonin prelabeled platelets in Hepes buffer. Total radioactivity was 34042 cpm in 0.5 ml of platelets suspension (5×10^7 platelets).

²⁾ Variability is given by $X \pm \text{SEM}$, data based on four determinations in each group.

³⁾ Significance was calculated by Duncan's multiple variability range test.

Different letters indicate statistically significant difference of values at $p < 0.01$.

Table 2 Effect of collagen hemostats on the ^{14}C -serotonin release by human platelets

Sample ¹⁾	^{14}C -Serotonin ²⁾ Release	Significance ³⁾	In % of Total Activity
Control (no fibers)	1545 \pm 40	a	4.5
Collagen fleece	8631 \pm 61	c	25.0
Fibrous form	3334 \pm 43	b	10.0

¹⁾ 10 mg of fibers were added to 2 ml of PPP and 8 ml of ^{14}C -serotonin prelabeled platelets in Hepes buffer. Total radioactivity was 24096 cpm in 0.5 ml of platelets suspension (5×10^7 platelets).

²⁾ Variability is given by $X \pm \text{SEM}$, data based on four determinations in each group.

³⁾ Significance was calculated by Duncan's multiple variability range test.

Different letters indicate statistically significant difference of values at $p < 0.01$.

magnesium phosphate and glucose (1 mg/ml). Final pellet was suspended in 35 ml of the buffer, and the count of platelets made.

3 ^{14}C -serotonin release

The final procedure for studying ^{14}C -serotonin release from labeled platelets consisted of using 10 mg of collagen sample (see **Table 1**) placed in 15 ml flat nalgene beakers. Control sample contained no collagen. Two milliliters of PPP and 8 ml of ^{14}C -serotonin prelabeled platelets in Hepes buffer was pipetted into beaker, stirred with a stir bar for five minutes, then transferred into nalgene round bottom centrifuge tubes and spun ten minutes at 2000 rpm. One-half milliliter of supernate of each sample was added to 9.5 ml Aquasol and counted in a Beckman scintillation counter Model LS-250.

Each sample was measured in quadruplicate analysis. Statistical evaluation of the results used Duncan's multiple variability range test to determine the significant differences among the tested groups.

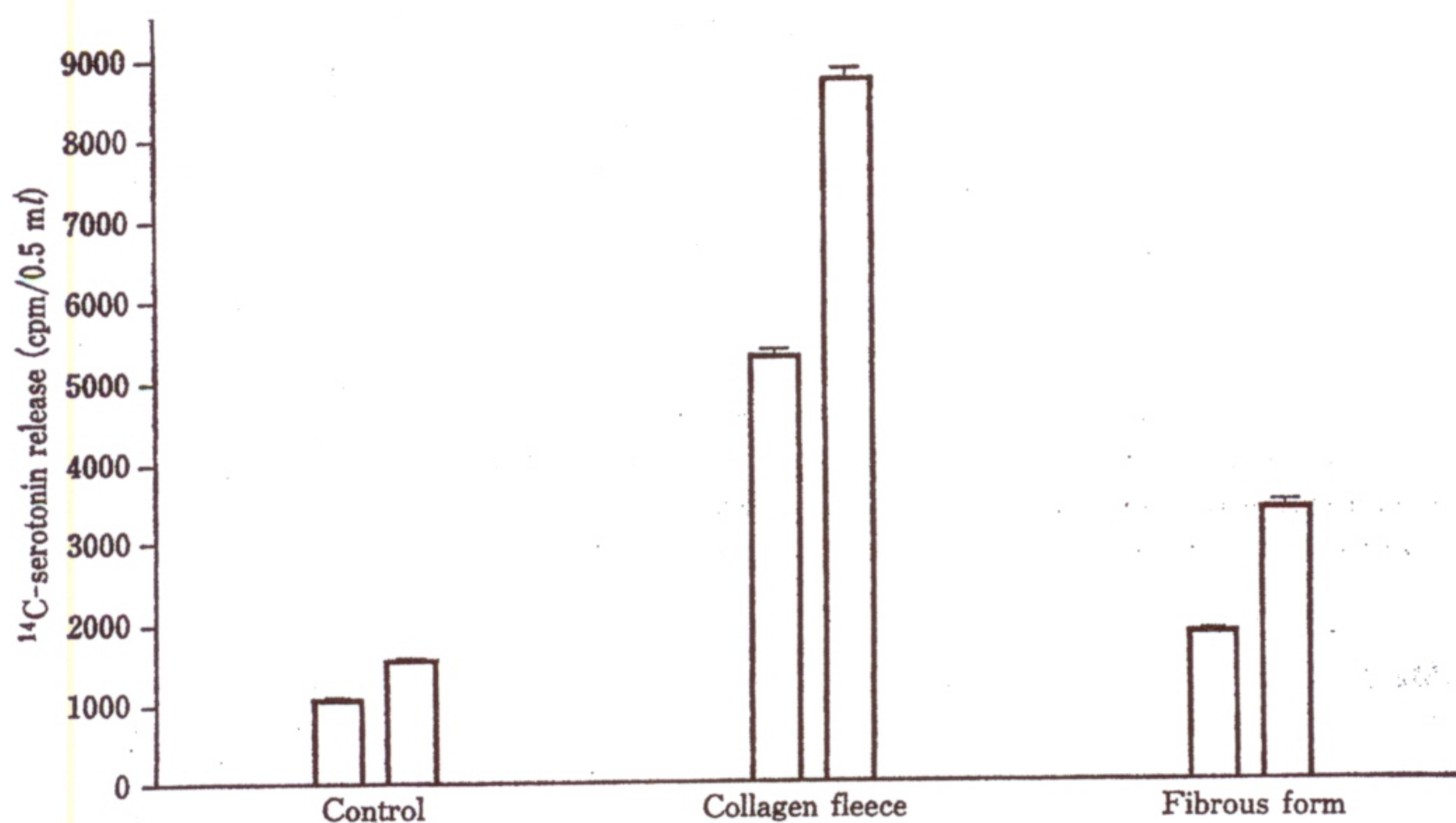


Fig. 1 Effect of collagen hemostats on the ^{14}C -serotonin release by human platelets
Data of two independent experiments are presented.

RESULTS

The results of two independent determinations of the potency of collagen fleece (Novacol) and fibrous form (Avitene) activate platelets and ^{14}C -serotonin release are presented in Table 1 and 2. There was a minimal variability of the results within each group, using four parallel determinations. The variation coefficient was always less than 2% of the mean value.

The presence of either collagen form in the incubation medium containing ^{14}C -serotonin prelabelled platelets resulted in a statistically significant increase of ^{14}C -serotonin released from platelets when compared to control samples without collagen. A significant difference between the effectiveness of fibrous form and collagen fleece was found with collagen fleece being more than twice as effective in promoting ^{14}C -serotonin release than the same amount of fibrous form.

The results of the two independent experiments were, in essence, the same. In the later experiment (Table 2), the platelets show higher fragility and susceptibility to collagen activation. Both experiments are summarized in Fig. 1, which best documents the above described findings.

DISCUSSION

Development of any collagen based hemostatic product goes through several steps, including determination of chemical purity, tissue reaction to the agent, rate of biodegradation⁸⁾, evaluation of antigenicity^{9,10)} and hemostatic effectiveness¹¹⁾.

Our preliminary tests with hemostatic collagen fleece, consisting of long and thin collagen fibers showed inconsistent results when working with the standard platelet aggregation test. Obviously, the nonhomogenous distribution of collagen fleece (even if segmented into smaller fragments) resulted in nonreproducible results. For this reason, we used ^{14}C -serotonin release to evaluate the activation of human platelets by two types of fibrillar collagen represented by Novacol and Avitene. Both of these commercial products have been on both the European and American market for a long time and the reports on their *in vivo* hemostatic effect state similar effectiveness^{12,13}. Some aspects favored fibrous form as to the hemostatic effectiveness, but not for handling characteristics¹⁴.

This study, performed strictly *in vitro* conditions, shows that collagen fleece promotes higher release of ^{14}C -serotonin from human platelets, prelabelled with this amine, than fibrous form. This discrepancy between the superiority of collagen fleece in *in vitro* tests reported and the clinical equivalence of collagen fleece and fibrous form is the best indication of the limits of the "*in vitro*" tests, when compared to the clinical results. One possible reason for higher effectiveness of one collagen fiber over the other may be the higher surface area of the fiber allowing for more efficient platelet adhesion. This does not seem to be the case with collagen fleece, which fibers are an average of 50 μm thick and several centimeters (0.5 to 4 cm) long. Fibrous form was reported to be 100 Å long fiber with a diameter 20–40 Å, thus indicating a higher surface area per weight unit. One possible reason may be the higher swelling (gelling) of collagen fleece, which has a lower structural stability of the collagen molecule when compared to fibrous form. However, it is not clear if the possible change of the viscosity of the incubation medium could affect serotonin release by platelets. It was documented that in *in vivo* animal models of controlled bleeding, the gelling of the topical collagen hemostatic agent contributed to the hemostatic effectiveness by "gluing" or mechanically obstructing the cut microvessels. Among other factors which might explain the higher effectiveness of collagen fleece fibers to enhance serotonin release from platelets is the type of collagen. It was documented that collagen type III in particular is a potent activator of platelets¹⁵. This, however, is not the case in this study as collagen fibers of both collagen fleece and fibrous form exclusively contain type I collagen of the same bovine origin.

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