Expression of P-selectin, TXA2, TGF-β1 and PDGF-AB in the Presence of Bioadhesive Chitosan Derivatives

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Abstract

Chitosan is a natural macromolecular polysaccharide, derived from chitin extracted from the exoskeleton of some marine invertebrates. This study was designed to evaluate the expression of P-selectin, thromboxane A2 (TXA2), transforming growth factor-β1 (TGF-β1) and platelet derived growth factor-AB (PDGF-AB) using enzyme-linked immunosorbent assays (ELISAs). The chitosan derivatives utilized were 7% N,O-carboxymethylchitosan (NO-CMC) (with 0.45 mL collagen), 8% NO-CMC, oligo -chitosan (O-C) and oligo -chitosan 53 (O-C 53). We found that O-C showed a significantly superior effect compared to NO-CMC in achieving hemostasis. Post-hoc multiple comparisons using Scheffe test indicated that p-values between the mean differences of all the assayed chitosan derivatives were not significant. However, our analyses successfully identified distinctions between the O-C and NO-CMC test groups and blood alone. These results may expand the understanding of the ability of chitosan derivatives to promote inflammatory responses and angiogenesis depending on their physical and chemical structures.

KEYWORDS: Chitosan, P-selectin, TXA2, TGF-β1, PDGF-AB

INTRODUCTION

In many hospital settings, maintaining a good hemostatic balance in bleeding patients remains a major challenge.[1] Successful approaches in hemostasis research may contribute to a significant reduction in hemorrhage related fatality. Among the novel hemostatic agents approved by the U.S. Food and Drug Administration (FDA), chitosan-based agents have shown great promise in preventing major hemorrhaging in the pre-hospital setting and in animal models of major blood loss.[2] Chitosan is derived from chitin, which can be extracted from the exoskeleton of some marine invertebrates. Chitosan is well

known for its potential as a non-toxic, biocompatible and biodegradable product.[3,4] Chitosan has the ability to expedite the wound healing process, arresting bleeding by facilitating platelet recruitments and promoting coagulation by forming a pseudo-clot. The chitosan structure can be chemically modified, and has been widely employed both as a biomaterial scaffold, for the controlled release of pharmaceuticals and as a component of successful wound dressing.[5,6] The compatability of chitosan biomaterials is a feature of sample preparation, viscosity, molecular weight, degree of deacetylation, incubation period and temperature. Although the study of chitosan in hemostasis has been the subject of intense research, to the best of our knowledge, few studies have been conducted on the effect of chitosan adhesion on platelet mediators. Therefore, this study measured the levels of P-selectin, thromboxane A2 (TXA2), transforming growth factor-β1 (TGF-β1) and platelet derived growth factor-AB (PDGF-AB). These mediators ease the activation of platelets to form the primary hemostatic plug and are involved in the rolling of platelets and neutrophils on activated endothelial cells promoting faster wound recovery.[7,8] We predict that varying formulations of chitosan- based hemostatic agents may have different potential roles in expediting hemostasis. In our research we used N,O-carboxymethylchitosan (NO-CMC) and oligo-chitosan (O-C) produced by the Standard and Industrial Research Institute of Malaysia (SIRIM Berhad) with degrees and acetylation between 75-95%. Our results showed that P-selectin, TXA2, TGF-β1 and PDGF-AB respond differently to the presence of chitosan derivatives with different molecular weights and degrees of deacetylation, which may facilitate the innovation of chitosan biomaterials to expedite hemostasis.

MATERIALS AND METHODS

Subjects

We recruited 80 healthy male and female donors aged 18 to 40 years and obtained written informed consent. Prior to commencing the study, ethical clearance was obtained from the Human Ethics Committee of Universiti Sains Malaysia (USM). None of the female donors were taking oral contraceptives when the blood samples were withdrawn. None of the healthy donors had been diagnosed with chronic diseases. Twelve milliliters of whole blood was withdrawn from the antecubital vein into three vials of ethylenediaminetetraacetic acid (EDTA) tubes for all the studies except for P-selectin. To evaluate P-selectin expression, three- way stop-cocks were used to collect blood under minimal tourniquet pressure, and the first 1mL of blood withdrawn was discarded. The remainder of each blood sample was aliquoted into 3 tubes containing 3.2% sodium citrate. Subject selection was contingent on a hematocrit level between 38% and 45% and a normal platelet count between 150 x 10^3/µL and 350 x 10^3/µL.

Materials

Chitosan sponges with variable chitosan formulations (7% NO-CMC with 0.45 mL collagen, 8% NO-CMC, O-C and one powdered type of chitosan termed O-C 53) were used. Lyostypt® was used as the positive control.

Methods

Blood samples were centrifuged at 1000 x g for 10 minutes. Supernatants were harvested and then centrifuged again at 10000 x g for 10 minutes. Chitosan samples, each weighing 10 mg, were dissolved in 50 µL of phosphate-buffered saline (PBS) (pH 7.4) and subjected to incubation at 37 ºC for 60 minutes. Five hundred microliters of platelet-rich plasma (PRP) was then mixed with each prepared chitosan sample for 30 minutes.[9-11]

Measurement of TXA2, P-Selectin, TGF-β1 and PDGF-AB

Levels of the above platelet mediators in serum upon adhesion to chitosans were measured using Quantikine colorimetric sandwich ELISA kits [TGF-β1 (Cat. No. DB100B), PDGF-AB (Cat. No. DHD00C) (R&D Systems, Minneapolis, MN, USA)]; P-selectin (Cat. No. CSB-E04708h), TXA2 (Cat. No. CSB-E09619h) (Cusabio Biotech Co., LTD)] according to the manufacturer's instructions. TGF-β1 was activated in the samples as directed in the assay protocol. Reactions were stopped, and absorbance was determined at 450 nm utilizing an ELISA reader (Tecan Infinite 200 PRO NanoQuant, Switzerland). A standard curve was generated, and the concentration of each sample was determined in ng/mL and pg/mL. Protein expression was calculated based on the volume of supernatant obtained after clot retraction. The standard curve was generated by plotting the absorbance for each standard on the y-axis against its concentration on the x-axis, and drawing a best-fit curve through all the data points.[12] No significant cross-reactivity or interference was observed among all the measurement levels.

Statistical analysis

All the data were presented as means with standard deviations. Statistical significance was evaluated using one-way analysis of variance (ANOVA) followed by post-hoc comparisons using Scheffe test. Statistical significance was defined as $p \leq 0.05$. Calculations were performed using SPSS version 18.0.

RESULTS

Expression of P-selectin

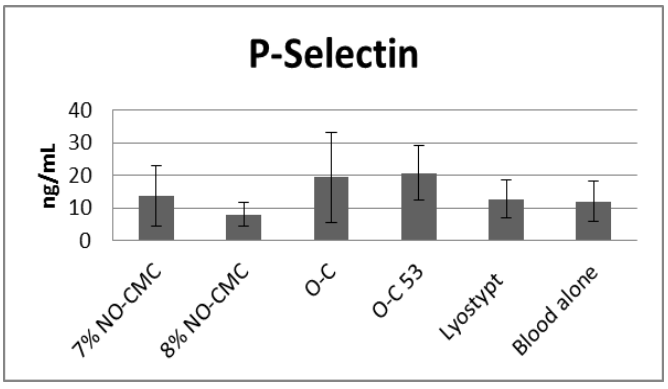

Fig 1. Expression of P-selectin

P-selectin was continuously expressed at low levels upon adherence to different forms of chitosan (Fig. 1). The highest expression level of P-selectin was induced by O-C 53 [20.71 (±8.50) ng/mL], followed by O-C [19.30 (±13.99) ng/mL]. Both O-C 53 and O-C increased 42.1% and 37.9%, respectively, compared to blood alone.

Expression of TXA2

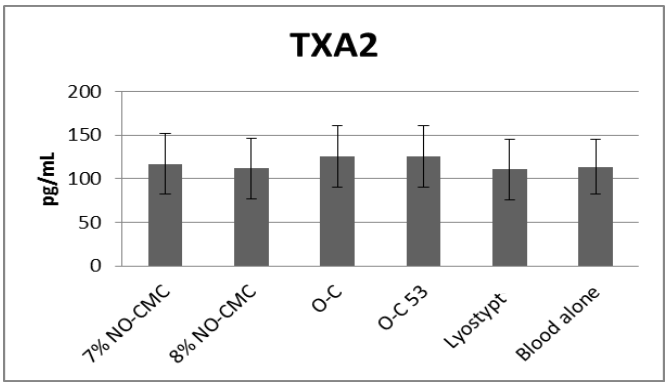

Fig 2. Expression of TXA2

The highest level of TXA2, [125.87 (±33.67) pg/mL] was released in the O-C group. O-C 53 expressed nearly equivalent level, differing by 0.5% from O-C [125.26 (±35.27) pg/mL]. Expression mediated by O-C represented an increase of 12.17 ng/mL compared to the blood alone group [113.70 (±31.56) pg/mL]. No significant differences were noted between the tested groups (Fig. 2).

Expression of TGF-β1

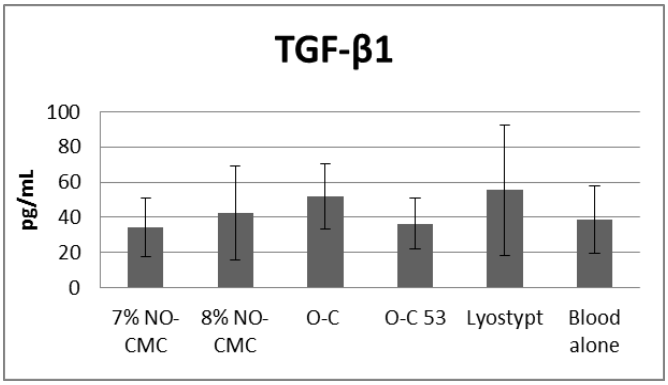

Fig 3. Expression of TGF-β1

The highest expression of TGF-β1 was seen with the Lyostypt control [55.62 (±37.0) pg/mL], followed by O-C [51.75 (±18.64) pg/mL] and 8% NO-CMC [42.28 (±26.72) pg/mL]. O-C and NO-CMC resulted in increases of 33.38% and 8.97%, respectively,

compared to blood alone. Lowered levels of TGF-β1 expression were observed with O-C 53 with [36.45 (±14.29) pg/mL] and 7% NO-CMC [34.20 (±16.69) pg/mL] similar to blood alone (Fig. 3).

Expression of PDGF-AB

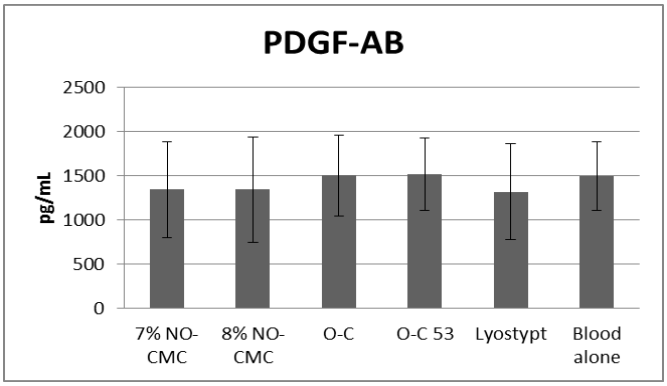

Fig 4. Expression of PDGF-AB

O-C 53 resulted in the highest concentration of PDGF-AB released [1513.18 (±411.73) pg/mL]. O-C also reached approximately the same level [1503.71 (±460.02) pg/mL]. The other chitosans registered low levels of PDGF-AB compared to blood alone [1496.35 (±392.34) pg/mL] (Fig. 4).

Expression	Comparison	Mean difference (95% CI)	p-value
P-Selectin*	7% NO-CMC	-1.73 (-10.92, 7.45)	0.995
	8% NO-CMC	4.03 (-5.15, 13.21)	0.818
	O-C	-7.31 (-16.50, 1.87)	0.210
	O-C 53	-8.72 (-17.91, 0.46)	0.074
	Lyostypt	-0.71 (-9.89, 8.48)	1.000
TXA2**	7% NO-CMC	-3.55 (-40.39, 33.29)	1.000
	8% NO-CMC	1.85 (-34.99, 38.69)	1.000
	O-C	-12.17 (-49.00, 24.67)	0.939
	O-C 53	-11.57 (-48.41, 25.27)	0.951
	Lyostypt	2.73 (-34.11, 39.57)	1.000
TGF-ß1***	7% NO-CMC	4.60 (-20.41, 29.62)	0.996
	8% NO-CMC	-3.48 (-28.50, 21.53)	0.999
	O-C	-12.95 (-37.97, 12.07)	0.689
	O-C 53	2.35 (-22.67, 27.36)	1.000
	Lyostypt	-16.83 (-41.85, 8.19)	0.399
PDGF-AB**	7% NO-CMC	154.74 (-377.02, 686.49)	0.964
	8% NO-CMC	152.02 (-379.74, 683.77)	0.967
	O-C	-7.36 (-539.11, 514.92)	1.000
	O-C 53	-16.83 (-548.59, 514.92)	1.000
	Lyostypt	178.97 (-353.78, 710.73)	0.934

Table 1. Comparison of means and p-values of tested chitosan –derivatives and blood alone

F-stat (df) = [6.23 (5), p<0.005*], [0.74 (5), p=0.594**], [2.74 (5), p<0.022***], [0.71 (5), p=0.615****]

The significant F-tests for P-selectin and TGF-β1 indicate that the mean values of the tested biomaterials are different between these groups (Table 1). At the same time, no statistically significant differences were noted in TXA2 and PDGF-AB expression, even between groups. Post-hoc multiple comparisons using Scheffe test indicated that the p-values between the mean differences of all the assayed chitosan –derivatives were not significant. However, as indicated by the negative values in the Table 1, differences in the means between the tested group and blood alone were noted for all of the factors evaluated. Our analyses successfully drew distinctions between tested groups (O-C, NO-CMC) and blood alone.

DISCUSSION

Chitosan is recognized as a natural polymer; it is a non-toxic, biodegradable, biocompatible and bacteriostatic polysaccharide. Various chitosan matrices have been studied and manufactured for wound dressings with bioadhesive properties.[13,14] A few studies reported that chitosan was able to induce intrinsic blood coagulation by inducing platelet adhesion, aggregation and activation[11, 15-17,20]. However, platelet aggregation is not the only correlate of successful blood coagulation.[11] Michael and Perumal reported that P-selectin expression stabilized the initial platelet aggregation formed by glycoprotein IIb/IIIa-fibrinogen interactions, permitting the accumulation of large aggregations.[15] P-selectin expression upon platelet activation has the potential to determine the size and stability of platelet aggregates, in addition to playing an important role in thrombosis.[18] In our study, P-selectin was continuously expressed at a low level upon adherence to the various forms of chitosan, with differences in the means observed between the tested groups. Investigation of P-selectin expression in PRP might be affected by mechanical platelet activation during centrifugation.[9] To minimize the possibility of mechanical platelet activation, three-way stopcocks were used to discard the first 1 mL of blood in the first syringe collected. Previously, it has been reported that the percentage of P-selectin induced by a chitosan-heparin composite scaffold was significantly reduced compared to that induced by a chitosan scaffold alone.[19] At the same time, levels of P-selectin were higher on platelets exposed to chitosan compared to platelets isolated from whole blood. The expression of integrin α2β3 has been reported to be elevated in platelets that adhered to chitosan.[16] This study produced results corroborating the findings of a great deal of our previous work in this field. We have reported that O-C affects thrombogenesis by changing the shape of platelets to pseudopodal, enabling aggregation.[20]

Although no significant difference in TXA2 levels was noted between the tested groups, the O-C group showed an increased release of TXA2. It is documented that TXA2 has the ability to trigger a multitude of different signaling cascades that regulate cellular ion flux,

cytoskeletal arrangement, cell adhesion, motility, nuclear transcription factors, proliferation, cell survival and apoptosis.[21,22,23] TXA2 plays a role in mediating the activation and recruitment of platelets to form the primary hemostatic plug. Apart from that, TXA2 is involved in stimulating platelet response to activate the hemostasis process and other immune responses.[24] O-C has the ability to trigger TXA2 by activating new platelets at the site of injury and by increasing platelet aggregation.

We investigated the release of TGF-β1 and PDGF-AB because both are important biochemical mediators in the wound healing process. Followed by Lyostypt, only O-C was able to elevate the expression of TGF-β1. O-C 53 showed decreased expression of TGF-β1. O-C and 8% NO-CMC are capable of causing the release of TGF-β1 from activated platelets and of stimulating the early stages of wound repair. These chitosans promote inflammatory responses and angiogenesis as well as the healing of acute and chronic wounds.[25-28] Previously, TGF-β1 was released from chitosan microgranules, which had been discovered to have a drug-releasing capacity, into bone defects during a 4-week testing period, and this treatment was found to accelerate bone regeneration and cell proliferation.[22] Senel et al., investigated the potency of TGF-β1 in the presence of a chitosan gel, which was found to be able to assist and promote the healing of the oral mucosa.[26] Our results are consistent with those of other studies, such as that of Okamoto et al., in which the authors concluded that chitosans induced the release of TGF-β1 and PDGF-AB from platelets.

PDGF has the ability to initiate dermal regeneration, elevate local protein and collagen synthesis and promote endothelial migration or angiogenesis during wound healing.[29-30] Their study highlighted that cytokine secretion was dependent on the degree of membrane injury in platelets.[11] This observation is consistent with our present study, where O-Cs were capable of inducing the expression of TGF-β1 and PDGF-AB. It is reported that solid-state chitosan has the ability to absorb platelets and accelerate hemostasis.[31] These results support the use of chitosan derivatives as a potential hemostatic agent for surgical and injury-induced wound healing.[11] The free NH_2 group in solubilized chitosan reacts with functional groups on the cell surface and promotes chitosan migration across the wound area.[27] From the previous finding, the extent of platelet activation in the presence of chitosan was found to be regulated by molecular weight, degree of deacetylation and addition of collagens. One of the reasons for the positive impact of O-C is its high degree of deacetylation (90%-95%). Chitosan films with a higher degree of deacetylation were found to be more brittle and harder to handle than those with a lower degree of deacetylation. The effect of degree of deacetylation on chitosan film biodegradation was also examined in rat tissues.[17] It was also observed that cell adherence and development were not affected by the degree of deacetylation, contradicting a previous study where better cellular attachment was observed with a higher degree of deacetylation.[18] However, our results suggest that degree of deacetylation, molecular weight and thickness of chitosan biomaterial play a crucial role in the absorption of platelets and in the expression of the factors studied. The purpose of this research paper was to study the expression of different blood-clotting mediators upon adherence to different chitosan derivatives. Our results raise many questions as to the mechanism of chitosan action following adhesion to blood cells. It is still not understood how and why chitosan biomaterials affect the release of the studied factors, other than the

potential influence imparted by chitosan properties such as degree of deacetylation, molecular weight, temperature, pH, viscosity and solubility. Although a considerable amount of literature has been published on chitosan as a hemostatic agent, the mechanisms of chitosan action are still under investigation. Future research should therefore concentrate on the investigation of physio- and bio-chemical alteration of chitosan biomaterials derived from chitin deacetylation. Further investigation on this topic needs to be undertaken before the effect of O-C and NO-CMC on hemostatic mechanisms is more clearly understood.

CONCLUSIONS

The most striking result that emerged from our present data was that O-C was superior to NO-CMC in activating platelets to form the primary hemostatic plug prior to coagulation. In our present assessment, an attempt has been made to understand the expression of P-selectin, TXA2, TGF-β1 and PDGF-AB in the presence of chitosan derivatives in various formulations. Because we used two different forms of O-C, we concluded that properties such as concentration, degree of deacetylation, roughness and thickness of chitosan derivatives are determinants of the enhanced hemostatic activity observed. Taken together, the current results are supported by our previous research reporting enhancement of platelet adhesion and aggregation by O-Cs. The relevance of these enhancements in platelet function was underscored by our current findings.

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