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The evaluation of resorbable haemostatic wound dressings in contact with blood in vitro

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Purpose: For many years research has been conducted on the development of resorbable, polymer, haemostatic materials designed to provide first aid and preliminary protection of injuries. The biological properties in vitro of a dressing in powder form called Hemoguard are expected to provide the ability to instantaneously stem bleeding with safe conditions of use. The aim of the study was to evaluate the haemostatic properties of a model of dressing based on micro- and nanofibrids of the chitosan, sodium/calcium alginate and/or carboxymethylcellulose complex. Dressings were prepared by spray-drying and freeze-drying. *Methods*: Human whole blood was subjected to timed contact with the haemostatic dressing model. Haemolytic action was determined by assaying the degree of haemolysis and evaluating blood cell morphology. Haemostatic action was determined on the basis of selected parameters of plasmatic clotting systems. *Results*: Dressings prepared by freeze-drying activated the coagulation system. The haemolytic index, plasma haemoglobin concentration values and blood cell morphological shapes were normal. Dressings prepared by spray-drying significantly activated coagulation. Activation of the coagulation process was evidenced by shorter clotting time of the plasma coagulation system and a longer process of clot formation. The dressing was associated with an increased haemolytic index and higher plasma haemoglobin concentration. The morphological shape of blood cells changed. *Conclusions*: The model of multi-resorbable wound dressings has haemostatic properties. The materials activate the clotting process more quickly than a single-dressing model. Increased activity was found for dressings prepared by spray-drying.

Key words: *haemostatic, dressing, chitosan-alginate micro- and nanofibrids, haemolysis, plasmatic clotting system*

1. Introduction

Products and materials that are used in the process of diagnosing or treating patients and that come into contact with the human body must have appropriate biological properties. The characteristics of medical products and materials are extensively tested in chemical, mechanical, and biological experiments in vitro and in vivo and in clinical trials. In planning experiments on medical products that are intended to come into contact with a patient's body, many factors should be taken into account regarding the suitability of intended use, potential toxicity, and the product's chemical, physical, electrical, morphological, and mechanical properties. Potential hazards associated with the use of materials can include short-term symptoms of acute toxicity, irritation, sensitization, and haemolytic or thrombogenic impact, as well as symptoms of long-term toxic effects that are chronic or sub-chronic, carcinogenic, teratogenic or related to sensitization or genotoxicity [21].

Treatment materials for injured tissues should not be cytotoxic or irritating, nor should they induce immunological reactions that have a negative effect on morphology and enzymes in the wound. Materials must adhere well to the wound, inhibit bleeding and stimulate regeneration of damaged tissues. A preferred feature is that dressing materials are biodegradable during wound healing and the process of

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removing a dressing does not damage sensitive tissue [10], [24].

For several years, there has been an increasing demand for dressing preparations that are mainly based on natural ingredients, convenient and easy-to-use. Additionally, the preparations should have basic protective functions that help to accelerate haemostasis, healing and the regeneration of damaged tissues. The extent of experimental testing on a given material should depend on the type of material, and the intended use or function as a medical product. Materials that facilitate local haemostasis use mechanical pressure on the bleeding site and also provide a structure for clot formation. A disadvantage of such materials is susceptibility to infection and the excessive staining of tissue caused by resorption [23]. Therefore, haemostatic materials with increased biocompatibility and improved haemostatic parameters are still being sought.

Polyaminosaccharides are appropriate components of dressing materials, especially chitosan and alginate due to their specific biological properties. Chitosan is obtained by the partial deacetylation of chitin, which is a component of the exoskeleton of crustaceans. Chitin and chitosan are non-toxic, biocompatible and biodegradable. They can be used in various forms and can undergo chemical or enzyme modifications. Materials are evaluated for medical use based on the biological activity involved in processes and phenomena such as: bacteriostaticity, biodegradation, adhesion, the impact on the membranes of living cells, and the natural compatibility with cells that result in the formation of a gel [16], [30]. Cationic chitosan and its hydrophilicity promote the process of wound healing, stimulate the formation of granulation tissue and re-epithelization, and reduce scar formation [18]. Both chitin and chitosan are widely used in medicine. One such use is as an artificial leather suture material (Chitosan Skin). Wound dressing materials made from chitin, Beschitin® (Urtica) Chitipack S® (Eisai Co.) and Chitipack P® (Eisai Co.), are used in the treatment of traumatic wounds. They have the ability to stimulate early granulation and prevent the formation of scar tissue.

Chitopack C® (Eisai Co.), which is made from chitosan that has a structure resembling cotton, is used to treat large traumatic wounds, especially those with a great deal of tissue loss. Formulations based on chitosan such as Tegasorb® (3M) and Vulnosorb® (Tesla-Pharma) are extremely useful in treating chronic wounds, ulcers and bedsores. An alternative formulation has collagen added. The use of polysaccharides in the preparation of bioactive haemostatic dressings and their use in clinical practice is an important direction in medical research [2].

There are a number of medical dressings on the market that inhibit bleeding that are made from chitin and chitosan derivatives. Syvek-Patch® (Marine Polymer Technologies) is made of microfiber chitin derived from diatom Thalassiosira fluviatilis, which is cultured under aseptic conditions. The preparation operates through the agglutination of red blood cells and the activation of platelets that aid in establishing a fibrin gel [17]. Dressing Excel Arrest® (LLC Haemostasis Co.) is also made from chitin. Alternatively, HemCon® (HemCon Medical Technologies, Inc.) ChitoFlex® (HemCon Medical Technologies, Inc.), ChitoSeal® (Abbott), Clo-Sur® (Scion Cardiovascular) are made of chitosan. HemCon® is freeze-dried chitosan, salt, and vinegar, and ChitoFlex® was improved in a convenient form with the same formulation. TraumaStat® (Ore-Medix) is a preparation made from lyophilized chitosan, polyethylene and highly porous silica. One advantage that TraumaStat® has is the very large surface that is achieved through the porous structure [3], [4], [6], [9], [20], [28]. Chitosan allows for the proper adhesion of the dressing into the vessels, and the silica activates internal coagulation [16]. Commercial dressings from RDH (Marine Polymer Technologies: Kaltostat® (ConvaTec), Melgisorb® (Mölnlycke), Sorbsan® (Maersk) SeaSorb® (Coloplast) and [8] use alginate, as non-woven, plate-shaped dressings for injuries on the skin surface or for deep wounds. The high adsorption and haemostatic features of the dressing material come from calcium ions that are released from the alginate into the wound, the activation of platelets and the acceleration of haemostasis. SeaSorb® additionally contains carboxymethylcellulose. Bandages are intended primarily for use in the first phase of treatment [15].

Tromboguard® (Tricomed SA) is a dressing material that is designed to provide first aid for traumatic wounds with great loss of connective tissue – especially in battlefield conditions – and for protection against external factors and preliminary protection against infection. The contact layer is a combination of active ingredients including: chitosan; calcium alginate; silver ions, which considerably reduce bleeding time and are located on a carrier that is a hydrophilic polyurethane sponge; and a semi-permeable membrane of polyurethane. The dressing can inhibit bleeding and protect the internal structure from the growth of microorganisms [13], [19].

Traditionally, the basic technique used to control blood loss has been continuous pressure on the wound. This enables blood clotting factors to accu-

mulate at the wound site, creates a mass of clotted blood, and inhibits blood loss. This technique, however, is not suitable for severe wounds and wounds with many points of bleeding, which can be a major cause of death. Bleeding to death is a specific risk on a battlefield, but it can also be a serious cause of death in trauma cases. Special dressing materials have been developed to effectively prevent the consequences of severe haemorrhage.

The Celox chitosan dressing was designed for potentially life-threatening bleeding episodes. Celox™ does not produce any heat, burn, or adhere to the wound. It is also effective in cases of hypothermia. The granular form provides a simple and fast way to control bleeding and it is especially useful in treating severe or deep wounds. There are several types of dressings sold under the Celox™ brand that differ in form. Celox is produced in three different sterile formulations: in granular form (Celox), as rolled gauze (Celox Gauze) and in a prefilled cylindrical applicator (Celox-A) [5].

Another rapid-acting agent for stemming life-threatening bleeding in mild or severe cases is QuikClot®. The product does not contain animal or human protein, which can cause allergic reactions. QuikClot® was originally produced as a granular zeolite and contained a special mixture of zeolite powder and mineral composites, which absorb water from the blood flowing from the wound and cause the blood to clot faster, while simultaneously producing heat. The active substance in the latest version of QuikClot ACS+™ is a naturally occurring, inorganic mineral called kaolin, which limits some of the reactive effects and produces a high temperature when in contact with the wound. It is made from a non-woven fabric, hydrophilic gauze impregnated with granules of kaolin. It is available as QuikClot® EMS Rolled Gauze and QuikClot Combat Gauze®, which are folded, soft, white bandages with kaolin. QuikClot® is not absorbed by the body and can cover the wound as long as necessary. QuikClot® should only be used in situations where conventional methods of bleeding control are ineffective [20], [28].

There has been an increasing demand observed lately in the medical supplies market for a new generation of haemostatic dressings for first aid and preliminary protection of battlefield injuries. These new generation dressings are expected to meet the demand for adequate haemostatic capacity in injuries and surgical wounds and antibacterial activity which prevents primary and secondary infections with safety of use.

For several years, the Institute of Biopolymers and Chemical Fibres (IBWCh), Łódź, has conducted research and development in the field of biomaterials that use biopolymers, in particular polysaccharides, for medical, pharmaceutical and veterinary medical purposes [11], [12], [29]. One of the key objectives of the research is to develop effective biodegradable, polymer, haemostatic materials. Methods have been developed for producing dressing preparations in a powder form called Hemoguard. The dressing material has been subjected to physicochemical and bacteriological evaluation [14]. The Department of Experimental Surgery and Biomaterials Research at the Medical University in Wrocław carried out biological evaluation in vitro.

The aim of this study was to evaluate the haemostatic properties of model dressings in powder form, made from chitosan/alginate Na-Ca and chitosan/alginate Na-Ca/CMC complex in the form of micro- and nanofibrids that could be used to produce modern dressings that inhibit bleeding in the first phase of local haemostasis. The new technology developed for dressing materials was targeted to the first phase of local haemostasis.

2. Materials and methods

2.1. Preparation of the dressing materials

The following materials were used in the study: chitosan: virgin chitosan (ChitoClear hqg 95, Primex ehf., Iceland), average molecular mass (Mv): 373 kDa, deacetylation degree (DD): 81%, ash content: 0.31%; sodium alginate (Protanal LF 10/60 FT, FMC Co.), sodium carboxymethylcellulose (Sigma-Aldrich Co.), calcium chloride anhydrous, analytically pure (POCh Co.); sodium hydroxide (Sigma-Aldrich Co.) and lactic acid 88% analytically pure (Avantor Performance Materials).

The chitosan/alginate Na-Ca complex and/or chitosan/alginate Na-Ca/CMC complex in the form of micro- and nanofibrids of Hemoguard were prepared according to the recommended IBWCh method [14], [19], using the Dispax Reactor Labor-Pilot 2000/4 flow reactor. The fibrid is a form of dressing prepared with chitosan (Chit) and alginate (Alg) measuring micrometer and nanometer, hence the name micro and nanofibrids. FDR – the abbreviation for fibrids; AlgNa-Ca – alginate, sodium calcium; CMC – sodium carboxymethylcellulose.

The model dressing symbols in the powder form: FDR/R/Chit/AlgNa-Ca, FDR/R/Chit/AlgNa-Ca/CMC, FDR/L/Chit/AlgNa-Ca, FDR/L/Chit/AlgNa-Ca/CMC.

The dressing material in powder form containing chitosan/alginate Na/Ca micro- and nanofibrids was prepared by two methods: freeze-drying (symbol L) with the use of the lyophilizer Alpha 2-4 LSC of Christ GmbH, Germany, and spray-drying (symbol R) with Spray Dryer B-290 from Büchi Co. The freeze-drying proceeded for 20 to 24 hours at temperature from –20 °C to +10 °C and vacuum from (0.1 to 0.7) mbar. The prepared dry micro- and nanofibrids were then disintegrated by means of the laboratory mill M-20, from IKA Werke. Conditions of the spray-drying: temperature of the drying head: 210 °C, temperature of circulating air: 95 °C, feeding rate 2.5–3 cm^3/min.

The dressings were sterilized in the Institute of Applied Radiation Chemistry. Irradiation dose was 25 kGy.

In the study model dressings in powder form were developed:

– FDR/L/Chit/AlgNa-Ca composed of chitosan 80%, AlgNa-Ca 20%, prepared by freeze-drying, a particle size of 10 μm–60 μm,
– FDR/L/Chit/AlgNa-Ca/CMC composed of chitosan 75%, AlgNa-Ca 15%, CMC 5%, prepared by freeze-drying, a larger particle size of 10 μm–60 μm,
– FDR/R/Chit/AlgNa-Ca composed of chitosan 80%, AlgNa-Ca 20%, prepared by spray-drying, a particle size of 0.9 μm–6.0 μm,
– FDR/R/Chit/AlgNa-Ca/CMC composed of chitosan 75%, AlgNa-Ca 15%, CMC 5%, prepared by the spray-drying, a particle size of 0.9 μm–6.0 μm.

Commercial haemostatic dressings tested were Celox™ (Med Trade Products Ltd., Cheshire, Great Britain) and QuikClot ACS+™ (Z-Medica Corporation 4 Fairfield Blvd., Wallingford, CT 06492). Celox™ products are made from chitosan, a substance obtained from shrimp shells in the form of powder with particle sizes of 0.1 mm–0.9 mm. QuikClot ACS+™ dressing is a nonwoven, fabric hydrophilic gauze impregnated with kaolin in the form of granulates with particle sizes 1.8 mm–2.0 mm.

2.2. Blood contact

The study was performed on whole human blood 0 Rh+ taken with liquid preservative fluid CPD (citrate-phosphate-dextrose). The consent of the Bioethical Commission of Wroclaw Medical University was granted for the study (No. KB-319/2013).

The biological testing of the model and the commercial haemostatic dressings was conducted by examining the haemolytic action and plasmatic activation of the coagulation system [1], [7], [22], [25]–[27].

2.2.1. Haemolytic activity assay

The volume and pH of the extract was measured. The total concentration of haemoglobin in the blood and citrate plasma and the supernatant was measured with the cyanmethaemoglobin method (van Kampena and Zijlstra) recommended by the International Committee for Standardization in Haematology [1].

Preparation of the calibration curve of haemoglobin

Standard haemoglobin (62.94 mg/cm^3) was diluted in seven dilutions in the range of 0.12 mg/cm^3–0.003 mg/cm^3 with the cyanmethaemoglobin reagent (Aqua-Med, Poland). For each concentration, absorbance at 540 nm was measured against the cyanmethaemoglobin reagent, then the calibration curve was plotted on graph paper.

Determination of plasma free haemoglobin

Blood citrate (CPD) with a volume of 3 cm^3 was centrifuged at 1018 g for 10 min. In each tube 0.5 cm^3 citrated plasma and 0.5 cm^3 of the cyanmethaemoglobin reagent was measured. After 15 min absorbance was measured with a spectrophotometer at 540 nm. A blank cyanmethaemoglobin reagent was used as a reference.

Plasma free haemoglobin (PFH) was calculated using the following formula

$$PFH = A^{PFH} \cdot F \cdot 2 \ [mg/cm^3] \quad (1)$$

A^{PFH} – absorbance, F – slope of the curve, or [(mg/cm^3)/A],

2 – dilution of the plasma.

The haemoglobin in the blood plasma tested did not exceed 2 mg/cm^3. The haemoglobin in the blood plasma tested was (0.69 ± 0.05) mg/cm^3.

Determination of total blood haemoglobin

In each test tube 5 cm^3 of the cyanmethaemoglobin reagent and 0.02 cm^3 (1:251) of whole blood (CPD) was measured. After 15 min absorbance of the solution was determined with a spectrophotometer at a wavelength of 540 nm. A cyanmethaemoglobin reagent was used as a reference.

The concentration of haemoglobin in the blood (C) was calculated with the following formula

$$C = A^{C} \cdot F \cdot 251 \ [mg/cm^3] \quad (2)$$

A^{C} – absorbance; F – slope of the curve, or [(mg/cm^3)/A],

251 – dilution of the plasma.

The total concentration of haemoglobin in the whole blood averaged (167.87 ± 3.83) mg/cm^3. To perform the haemolytic study the whole blood was diluted with DPBS to not more than 10 mg/cm^3.

Determination of haemoglobin in the diluted blood

In each test tube 4.5 cm^3 of the cyanmethaemoglobin reagent and 0.3 cm^3 (1:16) of diluted blood citrate (CPD) were dispensed. After 15 min absorbance at 540 nm with a thickness layer of 1 cm^3 was measured using a spectrophotometer. A cyanmethaemoglobin reagent was used as a reference. The concentration of haemoglobin in the diluted blood was read from the standard curve and multiplied by 13.5 to take into account dilution. The concentration of haemoglobin in the diluted blood (T) was calculated with the following formula

$$T = A^T \cdot F \cdot 13.5 \text{ [mg/cm}^3\text{]} \quad (3)$$

A^T – absorbance; F – slope of the curve, or [(mg/cm^3)/A],

13.5 – dilution of the plasma.

The concentration of haemoglobin in the diluted blood used for testing was not allowed to exceed 10 mg/cm^3.

Preparing the extract

Extracts of the test dressings were prepared in the proportion of 2 g/10 cm^3 of fluid extraction (DPBS – Dulbecco's phosphate buffered saline, Ca and Mg free, Lonza, Belgium) with an added volume of fluid for that absorbed by the material. A parallel test was done on an untreated control of a solution of DPBS without the dressing. Tests were incubated for 72 h at (37 ± 1) °C. Fluid extracted from the top of the material was used for the study.

Procedure for the test

In each test tube 3 cm^3 extract of the dressing material and 0.43 cm^3 of diluted blood were added. Simultaneously, a control solution of DPBS was prepared and incubated for 4 h at (37 ± 1)°C. Then, the test tubes were centrifuged at 656 g for 10 min to measure the haemoglobin concentration in the supernatant fluid from on top of the blood elements.

Determination of haemoglobin in the supernatant

In each test tube 1 cm^3 of cyanmethaemoglobin reagent and 1 cm^3 of the supernatant were added and after 15 min absorbance at 540 nm was measured. Markings were made in the supernatant absorbance of the test polymer solution and DPBS (control = blank).

The haemoglobin in the supernatant (S) was calculated with the formula

$$S = A^S \cdot F \cdot 2 \text{ [mg/cm}^3\text{]} \quad (4)$$

A^S – absorbance; F – slope of the curve, or [(mg/cm^3)/A],

2 – dilution of the plasma

Calculation of the haemolytic index

The calculated concentration of haemoglobin in the supernatant (S) and the concentration of haemoglobin (C) were used to determine the haemolytic index (I.H).

The index was calculated with the formula

$$\text{I.H.} = S \cdot 100/C. \quad (5)$$

The haemolytic index was calculated for the test dressings and the control solution of DPBS. The haemolytic grade was calculated from the difference between the middle-haemolytic index for the test sample and the control sample of the DPBS solution. The haemolytic grade was based on the calculated haemolytic index: 0–2 non-haemolytic, 2–5 slightly haemolytic and >5 haemolysis [1].

Morphological blood cells

20 µl of uncentrifuged test samples and controls of the suspension of blood cells were placed on a microscope slide and cover slipped. Morphological shape was evaluated using a light microscope at a magnification of immersion (1000×) for the unstained formulations of wet rated red blood cells, white blood cells or leukocytes, and the platelets. Images were documented photographically.

2.2.2. Coagulation tests

Tests were performed on samples of intermittent contact with human blood 0 Rh+ preservative fluid taken at CPD. The amounts of dressing material to blood volume were chosen experimentally. Human whole blood with samples of dressing materials were used in proportions of 0.003 g/3cm^3 and 0.003 g/2cm^3 and the control sample without dressing material was incubated at (37 ± 1) °C for 15 or 30 min. The whole blood was then centrifuged at 1467 g for 10 min and the plasma was separated from the weight of the cellular components of the blood. Platelet rich plasma was obtained for studying plasma coagulation and haemoglobin concentration [22], [25]–[27].

Research on plasma coagulation

Plasma was evaluated for partial thromboplastin time (APTT) and prothrombin time (PT). Thrombin time (TT) and fibrinogen (Fb), antithrombin (ATIII) and plasminogen (PLG) were also determined. Measurements of the plasma coagulation parameters were performed on a coagulometer Coag Crom 3003 at (37 ± 1) °C at a wavelength of 405 nm. The apparatus and reagent kits were from Bio-Ksel, Grudziądz, Poland. The procedures used were consistent with the methods contained in the manual for the camera.

Determination of time-activated partial thromboplastin

Test tubes were measured successively at (37 ± 1) °C with 50 µl of plasma and 50 µl of the APTT reagent, which was a buffered suspension of plant phospholipids mixed with silica, and incubated for 180 s at (37 ± 1) °C. Then, 50 µl of 25 mmol/L $CaCl_2$ was added and the plasma clotting time was automatically measured. The range of reference values of normal human plasma APTT was 26.00 s – 32.60 s – 38.90 s.

Determination of prothrombin time and fibrinogen

Test tubes were successively measured at (37 ± 1) °C with 50 µl of plasma, incubated for 60 s at (37 ± 1) °C, followed by the addition of 100 ul of thromboplastin (20 ± 1) °C, which was human placental calcium thromboplastin, and the plasma clotting time was automatically measured. The range of reference values for human plasma was PT, 9.90 s – 12.40 s – 14.90 s. The test was done to measure prothrombin time as an indicator of the quantity of fibrinogen. The range of Fb was 2.55 g/L – 3.19 g/L – 3.82 g/L.

Determination of antithrombin III

Test tubes were successively measured at (37 ± 1) °C with 50 µl of plasma (1:31) and 50 µl of thrombin (thrombin beef ash) and incubated for 60 s at (20 ± 1) °C, followed by the addition of a chromogenic substrate at (20 ± 1) °C. The test was done to measure clotting time as an indicator of antithrombin III activity. The range of AT III was 77% – 101% – 116%.

Determination of plasminogen

Test tubes were successively measured at (20 ± 1) °C with 50 µl of plasma (1:5) and 50 µl of streptokinase and incubated for 90 s at (20 ± 1) °C, followed by the addition of the substrate at (20 ± 1) °C and a measurement was taken. The test was used to measure clotting time as an indicator of plasminogen activity. The range of PLG was 77% – 92% – 107%.

Determination of the concentration of haemoglobin in the plasma

Test tubes containing 2 cm^3 of the cyanmethaemoglobin reagent had added to that 0.5 cm^3 of plasma diluted 5-fold. After 20 min absorbance of the samples and the standard cyanmethaemoglobin were determined spectrophotometrically at wavelengths of 540 nm and 680 nm. A blank cyanmethaemoglobin reagent was used as a reference. Tests were done in parallel.

The haemoglobin concentration of the plasma (Hbos) was calculated with the formula

$$Hbos = [(AB \cdot W) \cdot 5] / (AW)\ [mg/cm^3] \qquad (6)$$

where AB was the absorbance of the test sample = absorbance at 540 nm – absorbance at a wavelength of 680 nm; AW was the standard absorbance = absorbance at 540 nm – absorbance at a wavelength of 680 nm of the concentration of the standard in mg/cm^3. The haemoglobin concentration of plasma (Hbos) should be within the range of 5 mg/cm^3 to 40 mg/cm^3.

2.2.3. Plasma recalcification time test

Whole blood (CPD) was centrifuged at 1467 g for 10 min. After centrifugation, plasma was separated from the cellular components of the blood. A platelet rich plasma citrate was obtained. Dressing materials of 0.003 g were placed in a test tube, measured with 0.5 cm^3 citrated plasma and incubated for 15 min in a water bath at (37 ± 1) °C. Then, baguettes were inserted into a tube and 0.5 cm^3 of 25 mmol/L $CaCl_2$ was measured at (37 ± 1) °C. Test tubes were gently mixed and solidification time was measured. The measurement of clotting time (PRT) was completed at the time when the first fibrin strands connected the hook baguette with the test sample. Parallel measurements of plasma without dressing materials were done as a control [27].

2.2.4. Statistical analysis

The results were subjected to statistical analysis. An arithmetic mean (X), standard deviation (±SD) and the reference ranges (Min-Max) for the control group were calculated. The statistical significance of groups of treatment materials in comparison to the control group was determined with the T-test for independent samples with the use of Statistica 9.0. The correlation coefficients were assumed to be significant at $p < 0.05$.

3. Results

The haemolytic indices (H.I.) obtained for FDR/L/Chit/AlgNa-Ca (H.I.: 0.03), FDR/L/Chit/AlgNa-Ca/CMC (H.I.: 0.03) and QuikClot ACS+^{TM} (H.I.: 0.06) were within the normal range (0–2). For the dressing materials FDR/R/Chit/AlgNa-Ca (H.I.: 4.18), FDR/R/Chit/AlgNa-Ca/CMC (H.I.: 3.15) and Celox^{TM} (H.I.: 3.23) the haemolytic indices were in the range 2–5, which indicates a haemolytic grade level of: slight haemolysis [1].

Erythrocyte blood cells after incubation in a solution of DPBS (control) had a normal shape with short tab cytoplasm – echinocytes (Fig. 1a, b). Erythrocytes in the extract from FDR/L/Chit/AlgNa-Ca (Fig. 1c, d) and FDR/L/Chit/AlgNa-Ca/CMC (Fig. 1e, f) had a shape comparable to the control (Fig. 1a, b). Thrombocytes appeared singly or in small clusters and they exhibited slight adhesion or aggregation. In the extract from FDR/R/Chit/AlgNa-Ca (Fig. 1 g, h), FDR/R/Chit/AlgNa-Ca/ CMC (Fig. 1i, j), Celox^{TM} (Fig. 1k, l) as well as QuikClot ACS+^{TM} (Fig. 1m, p) the following observations were made: there were changes in morphology; erythrocytes and thrombocytes exhibited aggregation and mutual adhesion; there was adhesion of leucocytes to erythrocytes. Shades or membranes of erythrocyte blood cells were observed that were devoid of haemoglobin. Larger clusters of thrombocytes, and mutual adhesion and aggregation were observed in the extract from FDR/L/Chit/Alg Na-Ca. In contrast, a small amount of thrombocytes was observed in the extract from Celox^{TM} with erythrocytes that were morphologically changed. Erythrocytes in the extract from QuikClot ACS+^{TM} were morphologically changed and formed aggregates with clusters of thrombocytes. QuikClot ACS+^{TM} (Fig. 1o, p) in the form of powder showed increased adhesiveness and aggregation of thrombocytes in comparison to the granule form of the material.

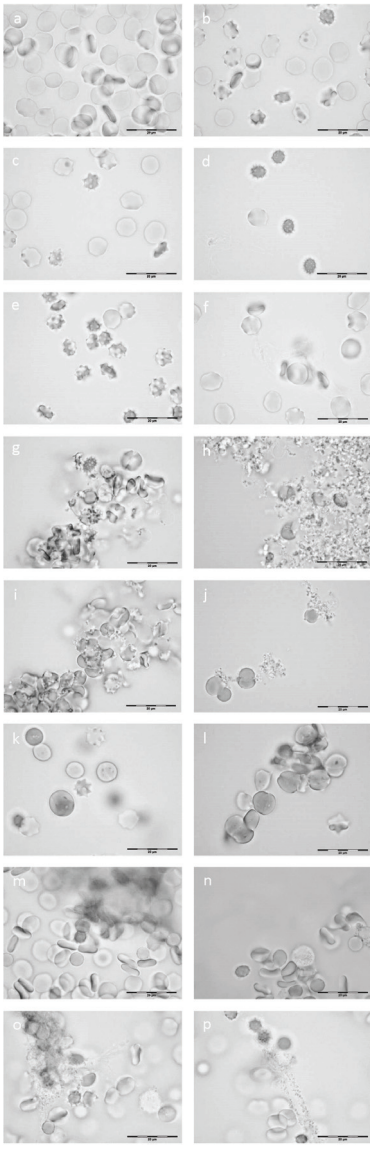

Fig. 1. Whole blood diluted after incubation 4h/(37 ± 1) °C solution DPBS – control (a)–(b), FDR/L/Chit/AlgNa-Ca (c)–(d), and FDR/L/Chit/Alg Na-Ca/CMC (e)-(f), FDR/R/Chit/AlgNa-Ca (g)–(h), FDR/R/Chit/AlgNa-Ca/CMC (i)–(j), Celox TM (k)–(l), QuikClot ACS+TM granules (m)–(n), QuikClot ACS+TM powder (o)–(p)

The haemoglobin concentration in the plasma (Hbos) dressings (0.003 g/3 cm^3) FDR/L/Chit/Alg Na-Ca, FDR/R/Chit/AlgNa-Ca and QuikClot ACS+^{TM} after 15 min and 30 min of contact with blood was within the normal range and did not exceed the value of 40 mg/dl. The FDR/L/Chit/AlgNa-Ca/CMC, FDR/R/Chit/AlgNa-Ca/CMC and Celox^{TM} after 30 min showed a significant (p < 0.05) increase in Hbos compared to the control (Fig. 2).

Using the proportions of 0.003 g/2 cm^3 there was a significant increase in Hbos in the FDR/L/Chit/AlgNa-Ca/CMC after 30 min (p < 0.01). There were no changes found for FDR/R/Chit/AlgNa-Ca/CMC and FDR/R/Chit/AlgNa-Ca after 15 and 30 min in which a clot occurred. After 15 and 30 min Celox^{TM} exhibited a significant (p < 0.01) increase in Hbos compared to the control with a value far in excess of the normal range. The FDR/L/Chit/AlgNa-Ca and QuikClot ACS+^{TM} did not show an increase in Hbos. The FDR/L/Chit/Alg Na-Ca and QuikClot ACS+^{TM} did not show a change in the Hbos (Fig. 2).

The activated partial thromboplastin time (APTT) found for FDR/L/Chit/AlgNa-Ca (0.003 g/3 cm^3) was comparable to the control group. For the FDR/L/Chit/AlgNa-Ca/CMC after 30 min there was a significant (p < 0.01) shortening of the APTT. For FDR/R/Chit/AlgNa-Ca/CMC and FDR/R/Chit/AlgNa-Ca there was a significant (p < 0.01) shortening of the APTT at

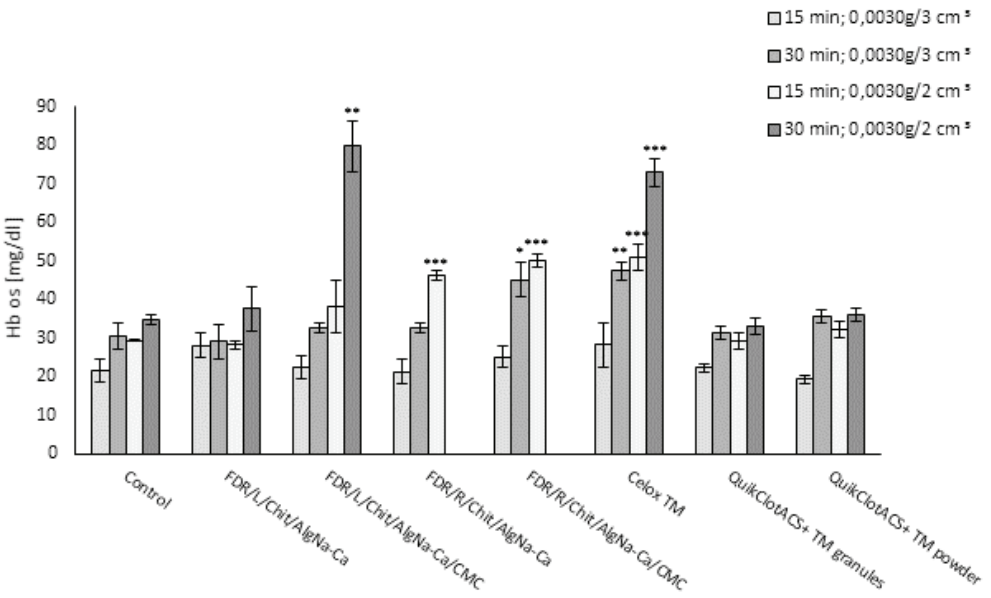

Fig. 2. The concentration of the haemoglobin in plasma (Hbos) and whole blood control after contact with the dressing at (37 ± 1) °C; reference range: (5–40) mg/dl
** $p < 0.01$, *** $p < 0.001$ – difference compared to control

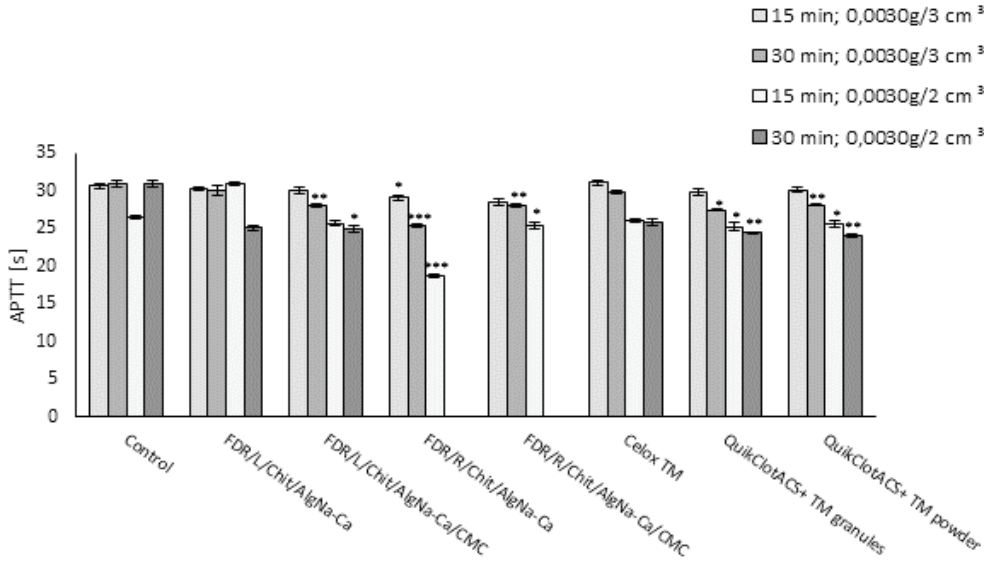

Fig. 3. The partial thromboplastin time (APTT) in the plasma of whole blood control and after contact with the dressing at (37 ± 1) °C
range (0.003 g/3 cm^3): 30.40 s–31.00 s (15 min), 30.40 s–32.60 s (30 min)
range (0.003 g/2 cm^3): 26.20 s–26.70 s (15 min), 25.10 s–25.70 s (30 min)
* $p < 0.05$,** $p < 0.01$, *** $p < 0.001$ – difference compared to control

0.003 g/3 cm^3 after 15 and 30 min and at 0.003 g/2 cm^3 after 15 min At 0.003 g/2 cm^3 after 30 min a clot did not occur. For the Celox™ there was no change in the APTT. For the QuikClot ACS+™ there was a significant ($p < 0.01$) shortening of the APTT (Fig. 3).

The prothrombin times (PT) found for FDR/L/Chit/AlgNa-Ca and FDR/L/Chit/AlgNa-Ca/CMC showed a significant ($p < 0.01$) shortening at 0.003 g/2 cm^3 after 30 min. For FDR/R/Chit/AlgNa-Ca/CMC and FDR/R/Chit/AlgNa-Ca there was a significant ($p < 0.01$) shortening of the PT at 0.003 g/3 cm^3 after 15 and 30 min and at 0.003 g/2 cm^3 after 15 min At 0.003 g/2 cm^3 after 30 min. there was clotting, which made it impossible to take a measurement. For the Celox™ dressing there was a significant ($p < 0.01$) prolongation of the PT and for QuikClot AC+™ a significant ($p < 0.01$) shortening of the PT (Fig. 4).

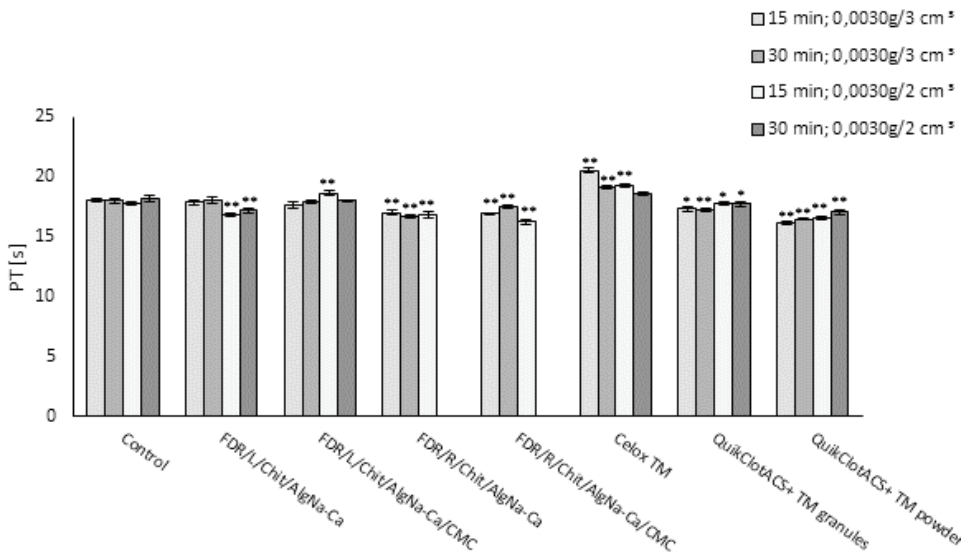

Fig. 4. The prothrombin time (PT) in the plasma and whole blood control after contact with the dressings at (37 ± 1) °C range (0.003 g/3 cm^3): 17.70 s–18.10 s (15 min), 18.00 s–18.30 s (30 min) range (0.003 g/2 cm^3):" 17.70 s–17.80 s (15 min), 18.00 s–18.50 s (30 min)
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ – difference compared to control

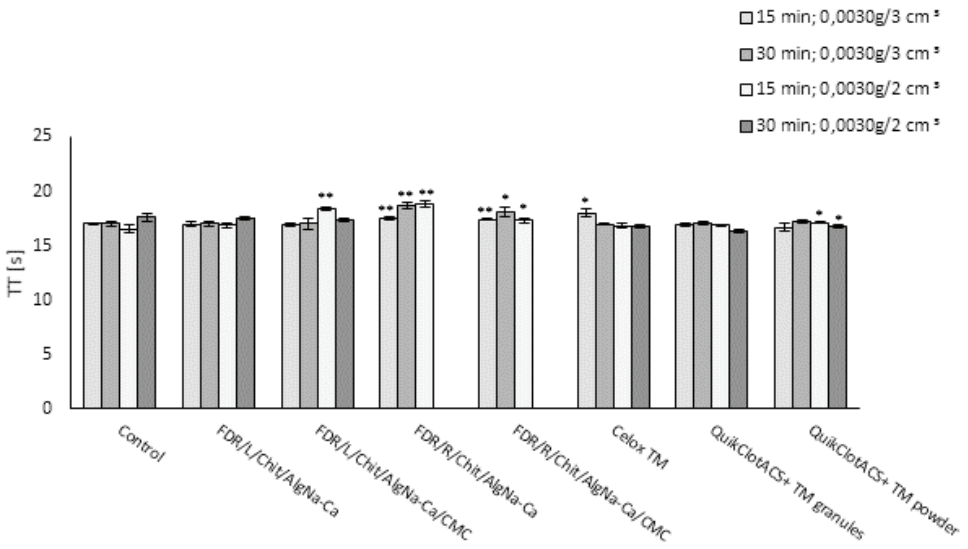

Fig. 5. The thrombin time (TT) in the plasma of whole blood control and after contact with the dressing at (37 ± 1) °C range (0.003 g/3 cm^3): 16.90 s–17.10 s (15 min), 16.80 s–17.20 s (30 min)
range (0.003 g/2 cm^3): 16.20 s–16.80 s (15 min), 17.10 s–17.90 s (30 min)
* $p < 0.05$ ** $p < 0.01$, *** $p < 0.001$ – difference compared to control

The thrombin time (TT) measured for FDR/R/Chit/AlgNa-Ca/CMC and FDR/R/Chit/AlgNa-Ca showed a significant ($p < 0.01$) prolongation at 0.003 g/3 cm^3 after 15 min and 30 min and at 0.003 g/2 cm^3 after 15 min. At 0.003 g/2 cm^3 after 30 min there was clotting and no measurement was made (Fig. 5). For other dressings the TT was similar to the control and reference values The fibrinogen concentration (Fb) ratio was 0.003 g/3 cm^3 after 15 min and 30 min, which was similar to the value in the control, while at 0.003 g/2 cm^3 after 15 min and 30 min, there was significant ($p < 0.01$) shortening of the Fb (Fig. 6). Activity of antithrombin III and plasminogen was within the normal range (Figs. 7, 8).

The plasma recalcification time (PRT) found for the FDR/R/Chit/AlgNa-Ca/CMC and FDR/R/Chit/AlgNa-Ca dressing was significantly shorter ($p < 0.05$) (Table 1A, 37% and 53%) in comparison to the control. The process of clot formation (PRT) was longer from the moment the fibrin thread appeared in the forming clot (Table 1A) until the clot formation was completed (Table 1B). For the Celox^{TM} addressing

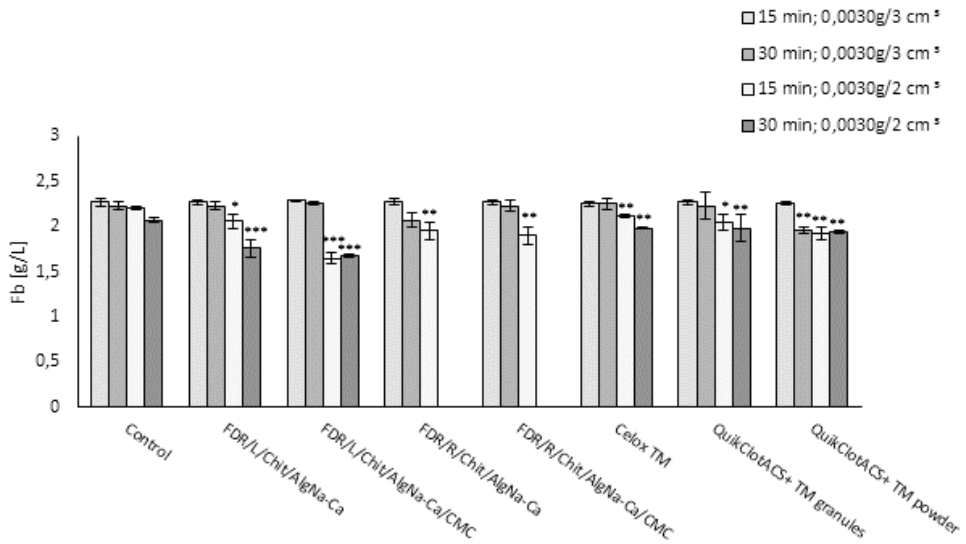

Fig. 6. The concentration of fibrinogen (Fb) in plasma and whole blood control and after contact with the dressing at (37 ± 1) °C
range (0.003 g/3 cm^3): 2.22 s–2.31 s (15 min), 2.17 s–2.28 s (30 min)
range (0.003 g/2 cm^3): 1.72 s–1.88 s (15 min), 2.10 s–2.07 s (30 min)
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ – difference compared to control

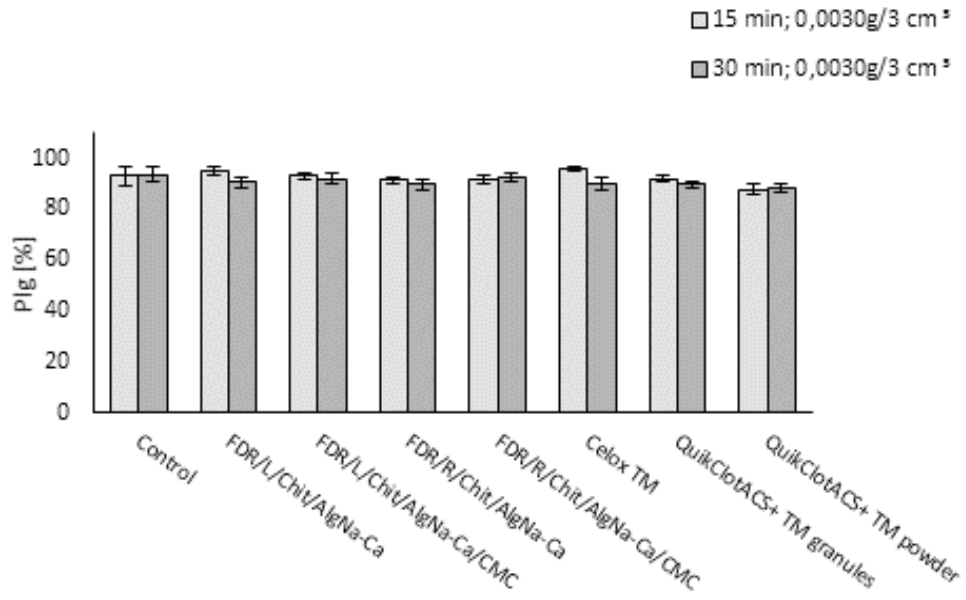

Fig. 7. The activity of plasminogen (Plg) in the plasma of whole blood after contact with the dressings (0.003 g/3 cm^3) at (37 ± 1) °C
reference range: 90.00%–96.00% (15 min), 91.00%–97% (30 min)

a fibrin thread appeared during clot formation in the control (Table 1A). However, the process of clot formation was longer (Table 1 B) in comparison to the control and comparable to the FDR/R/Chit/AlgNa-Ca/CMC and FDR/R/Chit/AlgNa-Ca dressings. The QuikClot ACS+^{TM} dressing in granular form had 78% shorter PRT (significance $p < 0.01$) and 86% shorter PRT in powder form compared to the control.

After samples of dressings in powder form were added to plasma observations were made macro- and microscopically (CKX 41, Olympus). The materials rapidly swelled and changed into an amorphous gel form. FDR/L/Chit/AlgNa-Ca (Fig. 9a, b) and FDR/L/Chit/AlgNa-Ca/CMC (Fig. 9c, d) formed a delicate, flake-like structure in the plasma, while FDR/R/Chit/AlgNa-Ca/CMC (Fig. 9e, f) and FDR/R/Chit/AlgNa-Ca (Fig. 9g, h) formed a compact dense mass. Celox ^{TM}

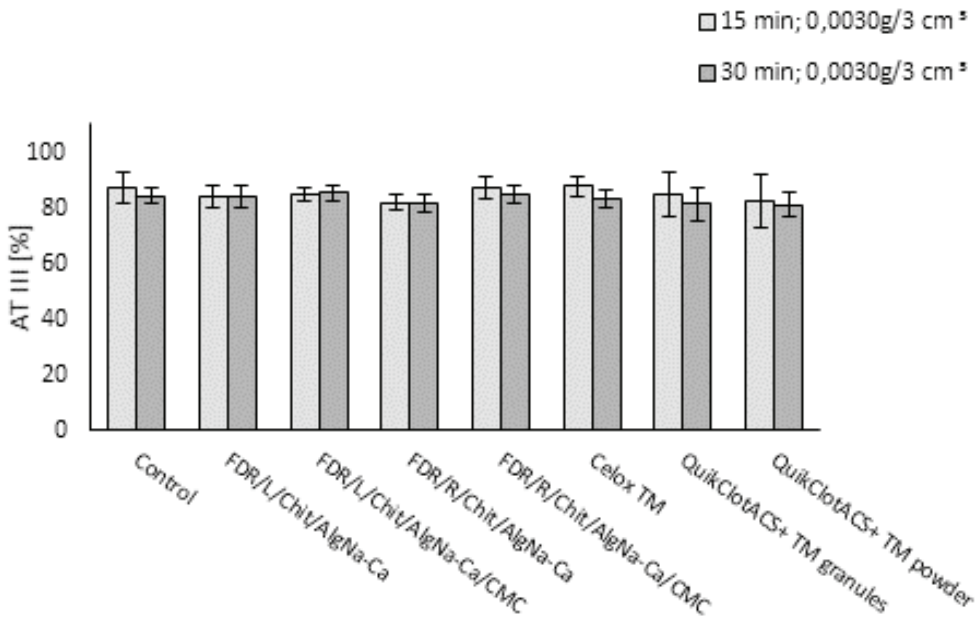

Fig. 8. The activity of antithrombin III (AT III) in the plasma of whole blood after contact with the dressings (0.0030 g/3 cm^3) at (37 ± 1) °C
reference range: 82.00%–93% (15 min), 81.00%–87% (30 min)

Table 1. Recalcification time citrated plasma (PRT) with dressing and the control plasma after 15 min. incubation at (37 ± 1) °C

No.	Dressing	PRT, s			
		A		B	
		X ± SD	*p*	X ± SD	*p*
1	FDR/LChit/Alg Na-Ca	466.67 ± 39.53	0.053	–	–
2	FDR/LChit/Alg Na-CaCMC	518.33 ± 30.51	0.219	–	–
3	FDR/RChit/Alg Na-Ca	270.66 ± 41.10*	0.013	456.00 ± 6.68*	0.019
4	FDR/RChit/Alg Na-CaCMC	363.66 ± 74.90*	0.017	652.66 ± 12.52*	0.045
5	Celox ™	540.00 ± 55.68	0.551	740.66 ± 35.75**	0.009
6	QuikClot ACS+ ™ granules	127.00 ± 25.81**	0.000	–	–
6a	QuikClot ACS+ ™ powder	80.00 ± 10.08***	0.000	–	–
K	Control +	568.33 ± 51.07	–	–	–

Reference range: 510 s–605 s, * $p < 0.05$, $p < 0.01$, *** $p < 0.001$ – difference compared to control.

(Fig. 9i, j) formed a structure of flakes with various shapes when in contact with plasma. After adding QuikClot ACS+™ (Fig. 9k, l) granules into plasma air bubbles were observed to have been released from the material. The granular and powder forms did not change shape macroscopically (Fig. 9m, n).

4. Discussion

Haemostasis is a set of processes aimed at maintaining blood in a liquid state in the vessels and, in the case of vascular injury, preventing extravasation, initially through the formation of platelets, and then, fibrin clotting. The disorder of haemostasis causes extravasation of blood, or the formation of blood clots within vessels. Disrupting the continuity of blood vessels causes blood to flow out until the time when the emerging haemostatic plug closes the damaged area. This process is called local haemostasis; it involves blood vessels and surrounding tissues, morphotic elements of blood, mainly platelets, and plasma coagulation factors. The efficacy of a haemostasis mechanism depends on an appropriate course of coagulation and fibrinolysis, proper adhesion and aggregation of platelets, and the state of the blood vessels. A well-functioning system of haemostasis may not be sufficient to stop blood loss. For this reason, a number of methods were introduced into

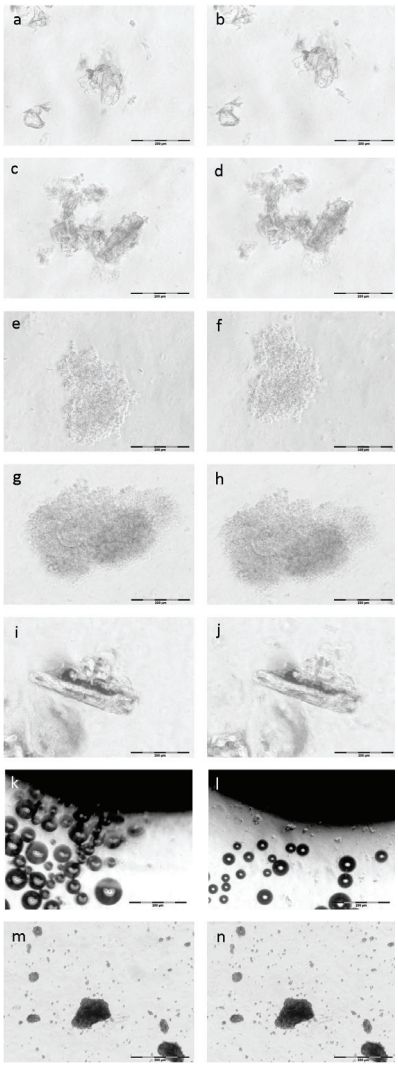

Fig. 9. The samples of dressings after filling into the plasma citrate: FDR/L/Chit/AlgNa-Ca (a–b), FDR/L/Chit/AlgNa-Ca/CMC (c–d), FDR/R/Chit/AlgNa-Ca/CMC (e–f), FDR/R/Chit/AlgNa-Ca (g–h), Celox TM (i–j), QuikClot ACS+ TM granules (k–l) and the powder (m)–(n) and after 15 min incubation in plasma. The image contrast microscopy – phase CKX 41 (Olympus)

medical use for the non-traumatic, haemostatic control of bleeding that acts locally [23].

In the living body blood is the most complex dynamic biological system. The individual components of blood ensure compliance with basic life functions, such as oxygen transport, removal of pathogens or repair of damaged tissues. Therefore, all medical products in contact with blood, directly or indirectly, must be biocompatible with blood.

This study was conducted to evaluate the haemostatic properties of new dressing materials in the form of powder named Hemoguard, which is a chitosan/alginate Na-Ca complex or a chitosan/alginate Na-Ca/CMC complex in the form of micro- and nanofibrids. The dressing materials were prepared by spray-drying (R) or with the use of a freeze-drying (L) and compared to standard dressing Celox™ pellets and QuikClot ACS+™ in granular and powder form. The evaluation was conducted on the basis of the haemolytic activity, the morphological observation of blood cells and the activation of the plasma coagulation system. The haemolytic activity of the dressing materials was determined by measuring the haemolytic index (H.I.) and plasma haemoglobin concentration (Hbos) and by observing morphological changes in the blood cells. The activation of the plasma coagulation system was determined by tests that measured APTT, PRT in the endogenous system, PT in the exogenous system and TT. The concentration of fibrinogen (Fb) was also determined.

QuikClot ACS+ ™ and Celox ™ dressings used in medicine are intended to stop life threatening bleeding of medium and large intensity and in cases of hypothermia.

QuikClot ACS+™ is a dressing made of a non-woven, hydrophilic gauze impregnated with kaolin, an inorganic mineral that is effective in stopping bleeding. It speeds up the body's natural coagulation cascade without causing a harmful exothermic reaction [3], [9]. The dressing is not absorbed by the body, does not stick to the wound, and is also easy to remove from the wound in hospital conditions. In this study of the QuikClot ACS+™ dressing in granular and powder form, there was aggregation of erythrocytes without haemolysis and increased activation of the endogenous system, as evidenced by a shortened APTT and PRT compared to the exogenous system with a shortened PT. The changes in the values of the parameters were significant with a larger amount of dressing material. The observed changes indicate that the materials contain components which accelerate the activation of the coagulation factors in plasma and these components participate in the process of clot formation.

The Celox™ dressing is in granular form and is made from chitosan with antibacterial properties. After applying it directly to a bleeding wound and pressing, the positively charged pellets of the dressing combine with the negatively charged blood cells to form a sticky pseudo-clot that blocks blood flow [3], [9]. The dressing does not initiate the normal blood coagulation process, it only causes the blood which is in direct contact with the dressing to bind. It is produced in granular chitosan with a known antibacterial agent which is not a coagulating agent. In our study on the Celox™ dressing the following observations were made when the dressing came in contact with whole blood: the aggregation of erythrocytes, changes in morphology, the aggregation of platelets, and mutual adhesion.

These changes caused haemolysis, as evidenced by the increase in the haemolysis index and haemoglobin concentration in plasma. An additional observation was the activation of the exogenous system by the extended PT without an impact on the endogenous system with normal APTT. The observed reduction in fibrinogen concentrations may have been the result of the activation of the fibrin monomers in the plasma that formed complexes with fibrinogen. The process of clot formation was extended in time from the fibrin thread appeared in the forming clot. The study confirmed that Celox^{TM} neither initiates the normal process of blood clotting, nor activates the endogenous system, but it does inactivate the exogenous system.

The abbreviated names of the dressing materials were used based on the method of production and composition of materials: FDR/L/Chit/AlgNa-Ca, FDR/R/Chit/AlgNa-Ca, FDR/L/Chit/AlgNa-Ca/CMC, and FDR/R/Chit/AlgNa-Ca/CMC. The differences in the composition of the dressing materials and methods of production for Hemoguard resulted in differences in the determined haemostasis values.

No changes were observed in the morphological shape of the erythrocytes, in the haemolysis index or in the concentration of the plasma haemoglobin when the FDR/L/Chit/AlgNa-Ca dressing came into contact with whole blood. With an increased amount of dressing material, there was a decrease in the fibrinogen concentration, and there was activation of coagulation in the endogenous system with a shorter PT without any observed impact on the exogenous system. For the FDR/L/Chit/AlgNa-Ca/CMC dressing the obtained values of the H.I. and Hbos were within normal ranges. With an increased amount of dressing material in the blood, there was an increased concentration of Hbos, the activation of haemostasis parameters with a shorter APTT and longer PT and TT and a decrease in the Fb concentration.

When the FDR/R/Chit/AlgNa-Ca and FDR/R/Chit/AlgNa-Ca/CMC dressing materials produced with the spray-dried (R) method came into contact with blood there was a larger degree of change in the values of the blood parameters: an increase in H.I. and Hbos, changes in the shape of the erythrocytes, and increased adhesion and aggregation of blood cells. There was activation of the haemostasis parameters with a shorter APTT and PT and a longer TT, as well as a decrease in the Fb concentration. The obtained values were slightly in excess of the range of referential values. With an increased amount of dressing material, there was clot formation which caused larger changes in the endogenous and exogenous system parameters, with an extended time for the process of full clot formation. The blood coagulation time in the presence of the dressing materials was shorter in comparison with other dressings. The whole process time of clotting was longer from the appearance of the first thread to the formation of the full clot. This has diagnostic significance because of the indication that the whole process of coagulation is activated when there is direct contact between the dressing material and the wound.

Much scientific research is being done to obtain dressing materials which stop bleeding quickly without side effects. The following characteristics of an ideal dressing material have been proposed: rapid arrest of arterial and/or venous bleeding with no thermal effect, biological indifference, non-toxicity, flexibility, adaptability to a wound that can be removed without leaving a residue, and stability in extreme temperatures. It should be cheap, effective in operation, safe for both the patient and supplier, and logistically feasible [8]. It is difficult to assign all the above features to one dressing currently available on the market.

On the basis of the results obtained in this research on Hemoguard resorbable dressings, it is possible to predict the haemostatic action when contact is made with a wound.

The Hemoguard dressing will come in powder form made of polysaccharides such as: chitosan, sodium/calcium alginate and/or carboxymethylcellulose. These added components and enhanced activity can contribute to accelerating the bleeding control process much more than with a single-component dressing.

The process of arresting bleeding in a wound comes from the electrostatic interaction of chitosan with blood cells, the presence of sodium alginate and cellulose with faster absorption of blood component secretions and increased stickiness and adhesiveness that keeps the formed gel clot from moving [25], [28]. The clot that forms is thus more stable. Sodium alginate and carboxymethylcellulose lead to high swelling capacity, the absorption of fluids and secretions and enhanced haemostatic properties, all of which is especially useful in dressing materials used in what is called moist wound therapy or in the treatment of wounds in the first phase of healing. In addition, the fibrous form of the dressing increases the contact surface and adhesiveness of the pseudo-clot that is in gel form. The interaction of several components in the dressing operates more effectively in the process of bleeding control than in one-component dressings. The process of activating haemostasis does not cause significant changes in the values of the coagulation factors involved in the complex coagulation process. This confirms that bleeding can be controlled by the fast reaction of the materials with the patient's blood,

in which blood components with unknown values of diagnostic parameters are not significant to the activation process.

The research conducted and the results obtained indicate that in vivo research should be conducted with multiple biodegradable Hemoguard dressings on various types of wounds. This will allow verifying the usefulness of these dressings under specific conditions.

5. Conclusions

1. FDR/L/Chit/AlgNa-Ca, FDR/L/Chit/AlgNa-Ca/CMC, FDR/R/Chit/AlgNa-Ca and FDR/R/Chit/AlgNa-Ca/|CMC dressings in contact with whole blood and plasma resulted in rapid absorption and changed the powder to an amorphous gel form, similarly to the reference Celox ™ dressing.
2. FDR/L/Chit/AlgNa-Ca and FDR/L/Chit/AlgNa-Ca/CMC dressings did not cause haemolysis, while FDR/R/ChitAlgNa-Ca/CMC and FDR/R/Chit/AlgNa-Ca caused slight haemolysis like the Celox™ dressing.
3. FDR/R/Chit/AlgNa-Ca, FDR/L/Chit/AlgNa-Ca, FDR/L/Chit/AlgNa-Ca/CMC and FDR/R/Chit/AlgNa-Ca/CMC dressings in direct contact with plasma rapidly activated coagulation compared to the Celox™ patch.
4. FDR/R/Chit/AlgNa-Ca dressing in the process of clot formation behaved like a Celox™ bandage, but the activation of the coagulation process was faster.
5. FDR/R/Chit/AlgNa-Ca/CMC and FDR/R/Chit/AlgNa-Ca dressings have similar haemocompatibility and increased haemostatic properties compared to the Celox™ dressing.

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References

[1] ASTM, F 756-08:2008. *Standard practice for assessment of hemolytic properties of materials*.

[2] CHENG Y.H., YANG S.H., LIU C.C., GEFEN A., LIN F.H., *Thermosensitive hydrogel made of ferulic acid-gelatin and chitosan glycerophosphate*, Carbohydr. Polym., 2013, 92, 1512–1519.

[3] DEVLIN J.J., KIRCHER S., KOZEN B.G., LITTLEJOHN L.F., JOHNSON A.S., *Comparison of chitoflex®, celox™, and quikclot® in control of hemorrhage*, J. Emerg. Med., 2011, 41(3), 237–245.

[4] ENGLEHART M.S., CHO S.D., TIEU B.H., MORRIS M.S., UNDERWOOD S.J., KARAHAN A., MULLER P.J., DIFFERDING J.A., FARRELL D.H., SCHREIBER M.A., *A novel highly porous silica and chitosan-based hemostatic dressing is superior to HemCon and gauze sponges*, J. Trauma, 2008, 65, 884–890.

[5] GEGEL B., BURGERT J., COOLEY B., MACGREGOR J., MYERS J., CALDER S., JOHNSON D., *The effects of BleedArrest, Celox, and TraumaDex on hemorrhage control in a porcine model*, J. Surg. Res., 2010, 164(1), 125–129.

[6] GUSTAFSON S.B., FULKERSON P., BILDFELL R., AGUILERA L., HAZZARD T.M., *Chitosan dressing provides hemostasis in swine femoral arterial injury model*, Prehosp. Emerg. Care, 2007, 11, 172–178.

[7] ISO 10993-4: 2009. *Biological evaluation of medical devices*. Part 4: *Selection of tests for interactions with b*lood.

[8] KHEIRABADI B.S., *Evaluation of topical hemostatic agents for combat wound treatment*, US Army Med. Dep. J., 2011, 2(1), 25–37.

[9] KOZEN B.G., KIRCHER S.J., HENAO J., *An alternative hemostatic dressing: comparison of Celox, HemCon, and QuikClot*, Acad. Emerg. Med., 2008, 15, 74–81.

[10] KRUCIŃSKA J., KOMISARCZYK A., PALUCH D., SZYMONOWICZ M., ŻYWICKA B., PIELKA S., *The impact of the dibutyrylchitin molar mass on the bioactive properties of dressings used to treat soft tissue wounds*, J. Biomed. Mater Res. Part B, 2012, 1, 11–22.

[11] KUCHARSKA M., STRUSZCZYK M.H., CICHECKA M., BRZOZA-MALCZEWSKA K., WIŚNIEWSKA-WRONA M., *Prototypes of primary wound dressing of fibrous and quasi-fibrous structure in terms of safety of their usage*, Fibres Text. East. Eur., 2012, 20(6), 142–148.

[12] KUCHARSKA M., CIECHAŃSKA D., NIEKRASZEWICZ A., WIŚNIEWSKA-WRONA M., KARDAS I., *Potential use of chitosan-based materials in medicine*, PCACD, 2010, 15, 169–176.

[13] KUCHARSKA M., NIEKRASZEWICZ A., CIECHAŃSKI D., WITCZAK E., STRUSZCZYK M.H., GULBA-DIAZ A., SUJKA W., *TROMBOGUARD® – nowoczesny opatrunek pierwszej pomocy (TROMBOGUARD® – modern first aid dressing)*, Mil. Phar. Med., 2010, 15(2), 22–28.

[14] KUCHARSKA M., WIŚNIEWSKA-WRONA M., BRZOZA-MALCZEWSKA K., STRUSZCZYK M.H., CICHECKA M., WILBIK-HAŁGAS B., RYBAK Z., SZYMONOWICZ M., PALUCH D., GUZIŃSKA K., KAŹMIERCZAK D., *Haemostatic, resorbable dressing of natural polymers-Hemoguard*, PCACD, 2015, 20, 130–141.

[15] LITTLEJOHN L.F., DEVLIN J.J., KIRCHER S.S., *Comparison of Celox-A, ChitoFlex, WoundStat, and Combat Gauze hemostatic agents versus standard gauze dressing in control of hemorrhage in a swine model of penetrating trauma*, Acad. Emerg. Med., 2011, 18(4), 340–350.

[16] MUZZARELLI R.A., *Chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone*, Carbohydr. Polym., 2009, 76, 67–182.

[17] PALMER B.L., GANTT D.S., LAWRENCE M.E., RAJAB M.H., DEHMER G.J., *Effectiveness and safety of manual hemostasis facilitated by the Syvek-Patch with one hour of bedrest after coronary angiography using six-French catheters*, Am. J. Cardiol., 2004, 93, 96–97.

[18] PIGHINELLI L., KUCHARSKA M., *Chitosan-hydroxyapatite composites*, Carbohydr. Polym., 2013, 93, 256–262.

[19] Polish patent appl. P.390253 *Opatrunek hemostatyczny warstwowy i sposób wytwarzania opatrunku hemostatycznego warstwowego* (*Multilayer haemostatic dressing and method to produce the dressing*).

[20] RAN Y., HADAD E., DAHER S., QuikClot Combat Gauze Use for Hemorrhage Control in Military Trauma: January 2009 Israel defense force experience in the Gaza strip – a preliminary report of 14 Cases, Prehop. Disaster Med., 2010, 25(6), 584–588.

[21] SZELEST-LEWANDOWSKA A., MASIULANIS M., SZYMONOWICZ M., PIELKA S., PALUCH D., *Modified Poly(carbonateurethane). Synthesis, properties and biological investigation in vitro*, J. Biomed. Mat. Res. Part A, 2007, 82(12), 509–551.

[22] SZYMONOWICZ M., FRĄCZEK-SZCZYPTA A., RYBAK Z., BŁAŻEWICZ S., *Comparative assessment of the effect of carbon-based material surfaces on blood clotting activation and haemolysis*, Diam. Rel. Mate.r, 2013, 40, 89–95.

[23] SZYMONOWICZ M., KRATOCHWIL J., RUTOWSKI R., STANISZEWSKA-KUŚ J., PALUCH D., *Evaluation of the influence of topical haemostatics materials on coagulation and fibrinolysis parameters*, Eng. Biomat., 1999, 7(8), 45–52.

[24] SZYMONOWICZ M., PALUCH D., SOLSKI L., PIELKA S., BŁASIŃSKA A., KRUCIŃSKA J., *Evaluation of the influence of dibutyrylchitin materials for activation of blood coagulation System*, Eng. Biomat., 2004, 7, 123–126.

[25] SZYMONOWICZ M., PIELKA S., OWCZAREK A, HAZNAR D., PLUTA D., *Study on influence of gelatin-alginate matrixes on the coagulation system and morphotic blood elements*, Macromol. Symp., 2007, 253, 71–76.

[26] SZYMONOWICZ M., RYBAK Z., FRĄCZEK-SZCZYPTA A., PALUCH D., RUSAK A., NOWICKA K., BŁAŻEWICZ M., *Haemocompatibility and cytotoxic studies of non-metallic composite materials modified with magnetic nano and microparticles*, Acta Bioeng. Biomech., 2015, 17(3), 49–58.

[27] SZYMONOWICZ M., RYBAK Z., WITKIEWICZ W., PEZOWICZ C., FILIPIAK J., *In vitro hemocompatibility studies of (poly(L-lactide) and poly(L-lactide-co-glycolide) as materials for bioresorbable stents manufacture*, Acta Bioeng. Biomech., 2014, 16(4), 131–139.

[28] WARD K.R., TIBA M.H., HOLBERT W.H., *Comparison of a new hemostatic agent to current combat hemostatic agents in a swine model of lethal arterial hemorrhage*, J. Trauma., 2007, 63, 276–284.

[29] WIŚNIEWSKA-WRONA M., KUCHARSKA M., KARDAS I., BODEK A., BODEK K.H., *Polymer biocomposites used in bedsores treatment*, PCACD, 2011, 16, 111–120.

[30] ZHANG J., XIA W., LIU P., CHENG Q., TAHI T., GU W., LI B., *Chitosan modification and pharmaceutical/biomedical applications*, Mar. Drugs, 2010, 8(7), 1962–1987.