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Chemical modification of implant vascular biomaterial. Part I. Optimization of gentamicin and ciprofloxacin modification conditions on vascular grafts to obtain antibacterial properties

Modyfikacja chemiczna wszczepiennych biomateriałów naczyniowych
Część I. Optymalizacja warunków modyfikacji protez naczyniowych gentamycyną
i cyprofloksacyną w celu nadania im właściwości przeciwbakteryjnych

Despite improvements in biomaterials, antibiotic prophylaxis and increasing surgical experience, a complete elimination of infectious complications after vascular procedures has not been achieved. When a prosthetic graft is infected, the therapy almost always requires its removal and replacement by autologous vein or prosthesis. Therefore, it is necessary to create infection–resistant vascular prostheses.

Several modifications to the basic graft have been proposed for improving its function. Vascular grafts covered with silver salts are currently applied [7]. Reduction of graft infections may be obtained by impregnation of commercial prostheses with antibacterial substances to prevent the bacterial adherence. Numerous research centers obtained this effect by antibiotics adsorption to prostheses sealed with gelatin, albumin or collagen [8]. Simple prosthesis soaking in antibiotic solution gives a short-term remedial effect because its antibacterial protection is limited to several days, depending on the antibiotic and biomaterial type [1, 13]. Thus, obtaining a biomaterial for vascular surgery with covalently bounding antibiotic, giving long-term remedial result is important.

Our target was to develop a new method of chemical binding of gentamicin and ciprofloxacin to vascular prostheses and as a result to obtain an effective and prolonged antibacterial protection of implants.

MATERIALS AND METHODS

The studies were conducted on Gentamicin sulphate (Polfa - Tarchomin S.A. Poland, 40 mg/ml) and Ciprofloxacin hydrochloride (Polpharma, Starogard Gdański, Poland). These antibiotics were immobilized on gelatin-sealed prostheses Uni-Graft® (Braun, Germany), made of polyethyleneterephtalate (PET), using three methods with the creation of 3 types of modified biomaterials. Additionally, standard curve for gentamicin was performed using standard Gentamicin sulphate 1mg/ml (Fluka). The microbiological control of antibacterial activity of antibiotic modified biomaterials was performed on plate tests with *E. coli ATCC 25992* strain. Supplementary antibacterial experiments of modified prostheses were continued on plate and liquid tests with use

Staphylococcus aureus ATCC 25923 (methicillin-sensitive), *Staphylococcus epidermidis* ATCC 12228 and *Escherichia coli* ATCC 25992 strains and these experiments were described in *Chemical modification of implant vascular biomaterial. Part II.*

IMMOBILIZATION PROCEDURE

Method I (carrier-Gentamicin) – covalently gentamicin immobilization on commercial prosthesis Uni-Graft® was described in the Polish Patent No. P-358934 [5]. This method was based on the procedure of protein-sealed carrier activation by bifunctional agent – glutaraldehyde (Fluka, Switzerland), with the formation of aldehyde groups on the surface. Antibiotic was further coupled to the prepared carrier by its free -NH_2 groups.

Method II (carrier-Ciprofloxacin) – ciprofloxacin impregnation on commercial prosthesis Uni-Graft® was performed by impregnation of biomaterial in ciprofloxacin solution. Washed up in 0.05 M phosphate buffer pH 7.0 pieces of graft (0.05g) were lightly dried and subsequently mixed with 1 ml of ciprofloxacin (2 mg/ml in deionized water). The whole mixture was mixed using a hematological shaker for 3 hours/22°C and lived for 10 hours/4°C. Temperature, time of impregnation and the kind of applied buffer (phosphate buffer pH 4.7, 5.7 and 7.0; glicyne buffer pH 3.6, 5.7 and 7.0; citrine buffer pH 3.6) were tested.

Method III (carrier-Gentamicin-Ciprofloxacin) – ciprofloxacin impregnation on covalently-modified prosthesis Uni-Graft® was performed by impregnation of covalently modified biomaterial in ciprofloxacin solution. Pieces of graft (0.05g) with gentamicin were washed up in 0.9% NaCl (200 ml) and H_2O (200 ml) and lightly dried (to leave on the carrier the covalently bounded genamicin only). They were subsequently mixed with 1 ml of ciprofloxacin (2 mg/ml deionized water). The whole mixture was mixed on a hematological shaker 3 hours/22°C and lived for 10 hours/4°C. Temperature, time of impregnation and the kind of applied buffer (phosphate buffer pH 4.7 and 7.0; glicyne buffer pH 3.6 and 7.0; citrate buffer pH 3.6) were tested.

ANTIBIOTICS DETERMINATION

1. Gentamicin sulphate concentration was quantitatively determined using the method of Cabanillas (2) with some modification [5]. Calculations of gentamicin concentration were performed using a standard curve for this antibiotic (Gentamicin sulphate 1mg/ml, Fluka) with the range 12.5–120 μg/ml. Amounts of antibiotic bound to support were calculated from the differences in gentamicin concentration in solutions before and after immobilization.

2. Water solutions of ciprofloxacin hydrochloride were directly spectrofotometrically measured at 272 nm. The concentration of ciprofloxacin in such samples was spectrofotometrically determined and calculations were performed using a calibration curve with Ciprofloxacin hydrochloride (Polpharma, Starogard Gdański, Poland) in the range 10.0–60.0 μg/ml. Amounts of antibiotic bound to support were calculated from the differences in ciprofloxacin concentration in solutions before and after immobilization.

RESULTS

Data shown in table 1 indicate that modification of vascular grafts with gentamicin and ciprofloksacin leads to create carriers bounded with these antibiotics. The optimal parameters of drugs immobilization process were obtained. The scale of immobilization process efficiency were

percentage yield and amount of antibiotic bounded with 1 g of carrier. It was shown that covalent immobilization of gentamicin (Method I, carrier-Gentamicin) occurred with an average yield 26%, which led to bound 0.6 mg of antibiotic with 1 g of prosthesis. Ciprofloxacin adsorption on commercial vascular carrier (Method II, carrier-Ciprofloxacin) as well as on early gentamicin modified prosthesis (Method III, carrier-Gentamicin-Ciprofloxacin) occurred with 8% yield, which led to bound average 0.15 mg of antibiotic with 1 g of prosthesis.

Table 1. Data (obtained from 16 repetitions) indicating gentamicin and ciprofloxacin immobilization effects

Carrier type	Gentamicin concentration before immobilization (mg/ml)	Ciprofloxacin concentration before immobilization (mg/ml)	Gentamicin concentration after immobilization (mg/ml)	Ciprofloxacin concentration after immobilization (mg/ml)	Gentamicin concentration on prosthesis (mg/g)	Ciprofloxacin concentration on prosthesis (mg/g)	Percentage yield (%)
1. carrier-G	2.44	0	1.79	0	0.65	0	26.1
2. carrier-C	0	1.94	0	1.77	0	0.17	8.8
3. carrier-G-C	2.58	2.04	1.97	1.903	0.61	0.14	Gentamicin 23.8 Ciprofloxacin 6.7

The possibility of antibiotics permanently binding on vascular biomaterial was displayed, so immobilization parameters optimization were needed. Although the gentamicin immobilization conditions were characterized in our patent, the ciprofloxacin immobilization conditions (Method *II,* Method *III*) were tested in this paper.

Buffer type for ciprofloxacin immobilization on Uni-Graft® prostheses was optimized at the beginning of our research. Because ciprofloxacin hydrochloride is not dissolved in alkaline buffers, other solvents were tested. It turned out that this drug is easily dissolved and can be adsorbed on gelatin-sealed prosthesis in acidicy medium. The scale of this process efficiency was not only analytical (percentage yield), but also had an antibacterial effect. Hence, the most satisfying was using redistilled water as a solvent (Table 2) on antibacterial character (37 mm of bacterial inhibition zone).

In further research, the influence of the drug immobilization time on the efficiency of this process was achieved. To obtain this effect, the standard reaction (Method *II*) of vascular prosthesis impregnation in antibiotic solution was controlled in time: 15 min, 1 h, 6 h, 24 h and 30h. The amount of ciprofloxacin bounded with prosthesis was analyzed after this time. Results of ciprofloxacin immobilization on matrix in dependence on time of this process are shown in Table 3. The data show that the most of antibiotic (0.16 mg/g) was bounded with a graft after 24-hours reaction. Shorter reaction times were not satisfying.

Table 2. Optimization of the type of medium for ciprofloxacin immobilization on PET prostheses (the average data obtained for 9 repetitions for Method II and Method III)

Medium pH	Ciprofloxacin adsorption immobilization yield (%)	Zones of *E. coli* growth inhibition (mm)
Phosphate buffer pH 4.7	7.8	28
Phosphate buffer pH 5.7	7.0	22
Phosphate buffer pH 7.0	5.6	21
Glicyne buffer pH 3.6	57.1	0.5
Glicyne Buffet pH 5.7	28.8	0.5
Glicyne buffer pH 7,0	4.7	0.2
Citrate buffer pH 3,6	crystalization after immobilization	-
Redistilled water pH 5,7	8.78	37

Table 3. Optimization of time of ciprofloxacin immobilization process on prostheses (the average data obtained from 12 repetitions for Method II and Method III)

Reaction time (hours)	Ciprofloxacin concentration before immobilization (mg/ml)	Ciprofloxacin concentration after immobilization (mg/ml)	Ciprofloxacin concentration on prosthesis (mg/g)	Percentage yield (%)
1	1.93	1.84	0.09	4.2
4	1.93	1.78	0.14	7.6
24	1.93	1.70	0.16	8.3
30	1.93	1.8	0.13	7.2

The influence of temperature of ciprofloxacin binding process on immobilization yield was also analyzed. To determine the optimal temperature of ciprofloxacin immobilization the standard reactions (Method II, carrier-Ciprofloxacin and Method III, carrier-Gentamicin-Ciprofloxacin) were conducted, but temperature was changed in four versions: in 4, 22, 37, 40°C. The results (Fig. 1) show that the optimal temperature range is 22–37°C.

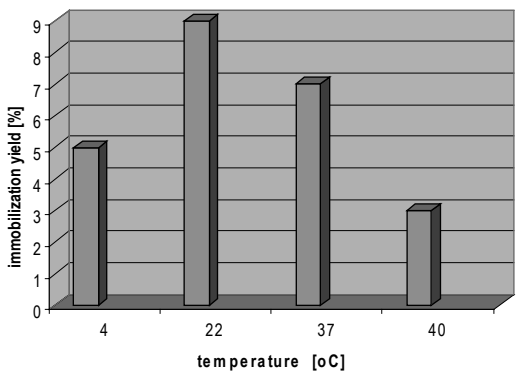

Fig. 1. The influence of the temperature of ciprofloxacin immobilization process on immobilization yield (the average data obtained from 9 repetitions for Method II and Method III)

DISCUSSION

Recent biomaterials research has been based on a vascular prosthesis simple soaking in antimicrobial drugs. For 10 years triclosan modified prosthesis have been implanted in a place of removed infected grafts. The new graft infection of these patients was not observed, but a systemic antibiotic therapy is needed [8]. Silver salts, which after bacterial DNA bounding block anelectrons transport in microbial cells are employed in antiseptic. This mechanism is safe and does not lead to bacterial resistance. However, antibacterial character was shown only by prostheses with high silver concentration and they caused an immunology reaction in a patient organism [3, 4]. These data are confirmed by Pupka, a scientist with the highest experience in such a kind of biomaterial among the Polish scientists. Because of a large danger with silver prosthesis application, its use only in a danger state of a patient life is recommended by Pupka [9, 11, 12].

In many research centers the experiments based on the creation and application of antimicrobial biomaterials have been made for years. In the beginning, synthetic prostheses were impregnated in antibiotics solution, but the drug was easily eluted, so this method was not efficient. The half-biological prostheses (albumin-, gelatin-, or collagen-sealed) bounded with antibiotic for elution time prolongation are employed now in vascular surgery. Simple impregnation of such PET-Dallon prostheses in antibiotic solution just before implantation is the easiest way of antibacterial modification. It was the most popular way to limit vascular biomaterials infection in recent years.

In vivo it was found, that antibiotic impregnated vascular grafts (specially in riphampicin) are much more resistant to bacterial infections than silver prosthesis [2, 7, 8]. Soaking biomaterials in proteins causes more efficiency ionic bounding with prosthesis (14 days) in comparision with not-proteinated biomaterials (3 days) [1, 7]. Although it was an easy and not expensive way of protection, it was still not sufficient. A lightly physically bounded antibiotic was eluted from prosthesis after a few days and bacterial colonization was possible.

The aim of our work was the drug binding to protein-sealed prosthesis not only with light physical bounds, but also with strong, covalent bounds. Therefore, we worked on antibiotic immobilization to gelatin-sealed vascular prostheses to obtain an effective and prolonged antibacterial protection of implants. We created a biomaterial hybride using two antibiotics (ciprofloksacin and gentamicin). It was obtained with two kinds of interactions: physical (a short-term antibiotic effect) for ciprofloksacin impregnation and chemical (covalent bounding giving a long-term result) for gentamicin immobilization. The recommendable research result is the creation of bacterial resistant hybride biomaterial. Such modified hybride biomaterials (carrier-gentamicin-ciprofloksacin) are protected from microbiological colonization (*Chemical modification of implant vascular biomaterial. Part B*). It seems that chemical modification does not influence antibiotics biological activity. Thus, biomaterial research should be intensified and continued.

CONCLUSIONS

Gentamicin covalent immobilization on gelatin-sealed vascular prosthesis is found out and leads to bound average 0.6 mg of antibiotic with 1 g of prosthesis. Ciprofloxacin adsorption on both a commercial vascular carrier and on a previously gentamicin modified prosthesis is possible and leads to bound average 0.15 mg of antibiotic with 1 g of prosthesis.

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SUMMARY

We worked on aminoglicosides and fluoroquinolons immobilization to gelatin-sealed vascular prostheses (made of polyethyleneterephtalate-PET-fibers) to obtain effective and prolonged antibacterial protection of implants. The optimal conditions of cyprofloksacin immobilization were determined. A new types of prostheses (carrier-Ciprofloxacin and (carrier-Gentamicin-Ciprofloxacin) were created.

STRESZCZENIE

Praca dotyczy immobilizacji wybranych aminoglikozydów i fluorochinolonów na żelatynowanych protezach naczyniowych (wykonanych z włókien polietylenotereftalanu – PET) w celu uzyskania efektywnej i przedłużonej w czasie ochrony przeciwbakteryjnej biomateriałów. Dobrano optymalne warunki unieruchomiania cyprofloksacyny na wyjściowych protezach naczyniowych (nośnik-cyprofloksacyna) oraz na protezach z wcześniej kowalencyjnie przyłączoną gentamycyną (nośnik-gentamycyna-cyprofloksacyna).