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Thrombin Generation by Gentamicin

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List of abbreviations

ΔA	Absorbance
AT-1	Antithrombin-1
AT-3	Antithrombin-3
Ca^{2+}	Calcium ions
CAT	Calibrated Automated Thrombography
CD11b	Cluster Determinant 11b (membrane receptor)
CRT	Coagulation Reaction Time
CS	Chromogenic Substrate
DIC	Disseminated Intravascular Coagulation
EXCA	Extrinsic Coagulation Activity Assay
F	Coagulation factor
IC	Inhibitory Concentration
IL	Interleukin
INCA	Intrinsic Coagulation Activity Assay
IU	International Units
K	Kallikrein
kDa	KiloDalton
LMWH	Low Molecular Weight Heparin
LPS	Lipopolysaccharide
MØ	Monocyte
Min	Minutes
MV	Mean Value
MW	Molecular Weight
NIC	Normal Intravascular Coagulation

PIC	Pathological Intravascular Coagulation
PK	Prekallikrein
PL	Phospholipids
PMN	Polymorphonuclear Neutrophil Granulocyte
PTT	Partial Thromboplastin Time
RECA	Recalcified Coagulation Activity Assay
SC	Stimulatory Concentration
SD	Standard Deviation
Serpin	Serine Protease Inhibitor
TF	Tissue Factor

Coagulation factors are expressed in Arabic numbers. In figures they may also be expressed in Roman numbers.

1.1. German Abstract

In der vorliegenden Arbeit wurde untersucht, wie die Blutgerinnung durch das in der Klinik oft verwendete Aminoglykosid-Antibiotikum Gentamicin (Gentamicin·H_2SO_4) beeinflusst wird. Die Grundüberlegung ist, dass Gentamicin als potentes Breitspektrum-Antibiotikum oft in lebensbedrohlichen, septischen Zuständen eingesetzt wird, in denen die physiologische Blutgerinnung bereits beeinträchtigt ist, im Sinne einer pathologischen, disseminierten, intravasalen Koagulopathie. Hier wurde untersucht, wie Gentamicin sich auf die Generierung des wichtigsten Enzyms der Hämostase, Thrombin, auswirkt. Dafür wurden 139 individuelle Citrat-Plasmaproben und 11 Citrat-Plasmapools gesunder Probanden verwendet. 50 µl Proben wurden mit Gentamicin in der klinisch relevanten Konzentration von 0-19.6 mg/l in Mikrotiterplatten supplementiert. Das Citrat-Plasma wurde unmittelbar danach rekalzifiert und das in der Gerinnungsreaktions-Zeit (coagulation reaction time = CRT) entstandene Thrombin im ultra-sensitiven, ultra spezifischen RECA (recalcified coagulation activity assay) quantifiziert. Diejenige plasmatische Gentamicin-Konzentration, welche die intrinsische Thrombin-Generierung ca. zweifach stimuliert, also die klinisch-relevante approx. SC200 (approximate 200% stimulatory concentration), wurde in der Gentamicin-Konzentration vs. Thrombin-Generierungs-Graphik ermittelt. 5 normale Plasmen wurden auch nach Zugabe von 0.5 IU/ml niedermolekularem Heparin untersucht. Bei der Auswertung zeigten 130 von 139 individuellen normalen Plasmaproben (94%) eine durch Gentamicin zweifach verstärkte Thrombingenerierung im Konzentrationsbereich von 2.0 ± 2.5 mg/l (MV ± 1 SD). 6 von 139 individuellen Plasmaproben (4%) zeigten sich gegenüber einer durch Gentamicin ausgelösten Thrombingenerierung resistent. Bei 3 von 139 individuellen Plasmaproben (2%) zeigte Gentamicin einen inhibitorischen Effekt auf die Thrombingenerierung und zwar mit approximativ 50% inhibitorischen Konzentrationen (approx. IC50) von ca. 1-2 mg/l.

Gentamicin aktiviert also die intrinsische Gerinnung mit großen inter-individuellen Unterschieden. Ein hämostaseologisches Monitoring und eine Testung der individuellen Empfindlichkeit der Gerinnung auf Gentamicin erscheinen unter einer

Therapie mit Gentamicin, insbesondere in einer koagulatorisch kritischen Situation, wie einer septischen Erkrankung, sinnvoll.

1.2. English Abstract

The present work examines how human coagulation is influenced by gentamicin (gentamicin·H_2SO_4), an often used aminoglycoside antibiotic in clinical practice. As a potent broad-spectrum antibiotic, gentamicin is frequently used in life-threatening, septic conditions, in which physiologic human coagulation is already affected in terms of an increased tendency to a pathologic disseminated intravascular coagulation. The influence of gentamicin on the generation of thrombin, the most important enzyme of human coagulation, was analysed. 50 µl samples of N=139 unfrozen individual normal platelet poor citrated plasmas and of N=11 unfrozen normal citrated plasma pools were supplemented with the clinically relevant concentration of 0 to 19.6 mg/l of gentamicin on microtiter plates. Instantly afterwards, the RECA (recalcified coagulation activity assay) was performed. The important approximate 200% stimulatory concentrations (approx. SC200) of gentamicin on thrombin generation were determined in the clinically relevant ascending part of the coagulation reaction time vs. thrombin generation curve. 5 normal plasmas supplemented with gentamicin as well as with 0 IU/ml or 0.5 IU/ml of the low molecular weight heparin enoxaparin-natrium were also analysed. 130 of 139 (94%) of the individual normal plasmas triggered thrombin generation with an approx. SC200 of 2.0 ± 2.5 mg/l (MV ± 1SD). Of the N=139 individual normal plasmas that were supplemented with up to 20 mg/l of gentamicin, 6 of 139 (4%) were resistant towards gentamicin-triggered thrombin generation. 3 of 139 (2 %) plasmas did not have an approx. 200% stimulatory concentration, but had an approx. 50% inhibitory concentration of 1-2.5 mg/l of gentamicin.

Gentamicin·H_2SO_4 triggers intrinsic coagulation and thus thrombin generation with great inter-individual differences. A hemostatic monitoring and testing of the individual sensibility to gentamicin is reasonable, especially in a critical pro-coagulant situation like sepsis.

2. Introduction

2.1. The human hemostasis

Hemostasis is the system of generation and destruction of thrombi. Didactically, human hemostasis is divided in 3 parts[90]:

1. Primary hemostasis
2. Secondary hemostasis
3. Tertiary hemostasis

Primary hemostasis is the system of the thrombocytes, secondary hemostasis is constituted of plasmatic coagulation and tertiary hemostasis represents fibrinolysis. This didactical classification does not exactly reflect the biologic processes, but it is chosen due to a simplified representation.

Pathophysiologically or clinically, secondary hemostasis is often the most important part of hemostasis, because many diseases via cell fragments directly start with activation of secondary hemostasis with its protease cascades, autocatalytic amplifications, and phospholipid-enhancements[70].

Physiologically, hemostasis often starts with activation of primary hemostasis. A break in the vasculature exposes extracellular matrix (collagen molecules) to flowing blood and initiates the coagulation process. Platelets adhere to the site of injury through a number of specific interactions[42] mainly through collagen of the basement membrane and associated adhesive proteins[69]. Von Willebrand Factor (vWF) binds exposed collagen and, after a conformational change, binds platelets through the platelet receptor glycoprotein-(GP-)Ib-IX-V complex[42] and activates primary hemostasis[16].

After adhesion platelets undergo cytoskeletal changes and become stellated by developing pseudopodia in order to cross-link. Moreover, they release calcium ions, ADP, serotonin, and thromboxan A_2 from their granula[86]. Thromboxan A_2 has vasoconstrictive properties[93]: it reduces the blood flow rate and stops the bleeding.

The first clot develops; the cross-link of platelets leads to a primary wound closure and thrombin is generated.

The result of human plasmatic hemostasis is the formation of fibrin generated by thrombin during the three phases of plasmatic coagulation: initiation, amplification, and propagation[101]. Fibrin is stabilized via gamma-gamma dimerization (cross-linking) of its D-subunits by factor 13a.

Plasmatic hemostasis is divided in an extrinsic and in an intrinsic pathway.

The extrinsic pathway is also called tissue factor pathway, because the injured cell (often monocytes or fibroblasts) releases tissue factor (TF), which activates coagulation factor 7 to factor 7a. F7a-TF-PL-Ca^{2+}, the extrinsic ten-ase, activates factor 10; factor 10a converts prothrombin (factor 2) into its active form, thrombin (factor 2a).

The intrinsic pathway is also called contact activation pathway, because a changed surface folds factor 12 to factor 12a (F12a) or pre-kallikrein (PK) to kallikrein (K). Factor 12a and kallikrein generate each other; F12a activating pre-kallikrein and kallikrein activating Factor 12. Factor 12a and kallikrein activate factor 11. Factor 11a activates factor 9 to factor 9a (F9a); then F9a-F8a-PL-Ca^{2+}, the intrinsic ten-ase, activates factor 10 to factor 10a[55].

In the final common pathway factor 10a in complex with F5a-PL-Ca^{2+} converts prothrombin into thrombin. Thrombin converts fibrinogen into fibrin. Factor 13a is generated by thrombin and cross-links a D-gamma chain to another D-gamma chain, stabilizing the fibrin clot.

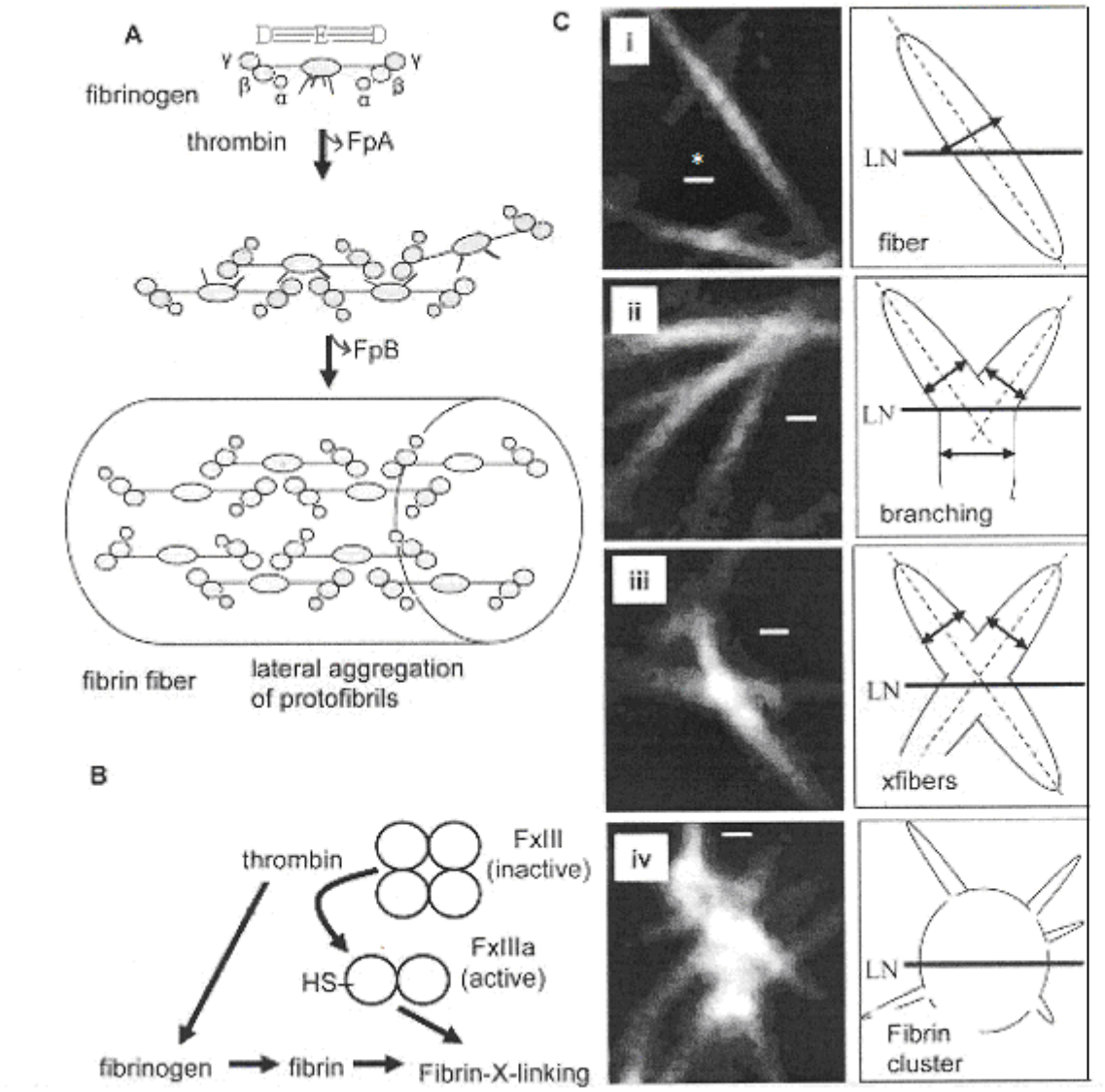


Figure 1

Conversion of fibrinogen into fibrin by thrombin. Reproduced with permission of PLoS One[7] ($\overset{*}{-}$ =0.7 μm).

Panel A. Conversion of fibrinogen into fibrin. The fibrinogen molecule is composed of three pairs of polypeptide chains. Thrombin splits the fibrinopeptides A (FpA) and B (FpB) from the central domain E. Domain D and E interact leading to aggregation of protofibrils; fibrin is generated.

Panel B. Schematic representation of thrombin converting fibrinogen into fibrin. Cross-linking of fibrin is induced by factor 13a which is activated by thrombin.

Panel C. Multiphoton images of fibrin. Fibrin fibers (i), fiber branch junctions(ii), crossing fibers (iii) and fibrin clusters (iv)[7].

2.2. The protease thrombin

Thrombin is the most important enzyme of human coagulation[81]. Thrombin is synthesised as prothrombin in the liver. It is a serine protease and it is generated from its inactive precursor prothrombin upon intrinsic or extrinsic coagulation activation[85]. Prothrombin binds together with calcium ions to the negatively charged phospholipid membranes. It also binds to the macromolecular enzyme complex prothrombinase (factor 10a, 5a, Ca^{2+}, and phospholipids). This converts prothrombin into the active enzyme thrombin[11]. Thrombin activation *in vivo* is a very complex procedure. The activation reactions are divided in three phases: initiation, activation, and propagation phase[15]. In the activation phase, a matrix change of blood generates Factor 12a and kallikrein or active tissue factor binds factor 7. The resulting complexes (the intrinsic ten-ase F9a-F8a-PL-Ca^{2+} or the extrinsic ten-ase F7a-F5a-PL-Ca^{2+}) activate factor 10. The amplification phase is characterised by factor 10a or kallikrein converting small amounts of prothrombin to thrombin which activates cofactors 8, 5, and platelets.

The most important hemostatic function of thrombin is to convert fibrinogen into fibrin[85]. On this way thrombin is the main enzyme of plasmatic hemostasis. Because of factor 5 and 8 activation during the amplification phase, thrombin indirectly catalyses its own generation in terms of a positive feedback mechanism[68]. Factor 5 and 8 are therefore very important for thrombin generation[10]. Active thrombin is composed of two polypeptide chains connected through a disulfide bond. Polypeptide chain A consists of 36 amino acids and polypeptide chain B of 259[59]. Recent studies demonstrated how thrombin concentration biochemically influences the growing thrombus in vivo, determining fiber thickness, density, structure, rate of formation and stability of the fibrin clot[101]. Abnormal thrombin activities and a consequential abnormal fibrin clot increases the risk of insufficient fibrinolysis[17].

Thrombin in a side reaction can also bind to thrombomodulin. The thrombin/thrombomodulin complex activates protein C. Activated protein C destroys

coagulation factors 5a and 8a. Therefore thrombomodulin can shift the activity of thrombin towards anticoagulation. Based on the membrane binding and activation steps, thrombin should only be released into the local region of injury and bleeding. However, in pathophysiology high activities of thrombin often gain access to the systemic circulation causing a change from normal to pathologic intravascular coagulation (NIC → PIC).

The enzyme activity of thrombin is controlled by different inhibitors, especially by the serpin antithrombin-3 (AT-3), which is the major inactivator of thrombin in human blood. Additionally, the serpin heparin cofactor 2 is a minor inactivator of thrombin. The broad spectrum inhibitor alpha$_2$-macroglobulin constantly complexes about 10% of thrombin generated in blood, the complexed thrombin is still amidolytically active; it is rather transported than inactivated. The serpins binding is enhanced by polysulphated glycosaminoglycans such as heparin. Hirudin, a polypeptide coagulation inhibitor produced by the leech, inhibits thrombin by interaction with its active centre[59]. Fibrin can also inhibit thrombin: thrombin gets entrapped in generated fibrin (antithrombin-1 = AT-1)[58].

Thrombin also has an effect on different cell types not directly related to hemostasis, but to inflammation, proliferation, or reparative response to wounding[85]. On this way thrombin is an important inflammatory mediator. It is reported for thrombin to enhance T lymphocyte and monocyte activation and induce the release of proinflammatory cytokines such as interleukin 6 and interleukin 8. Furthermore, thrombin inhibits the release and the expression of IL-12 in human peripheral blood mononuclear cells. Such inhibition is accompanied by enhancement of IL-10 release. This mechanism could be responsible for increased complications in immunosuppressed patients after an injury[62]. Furthermore, thrombin seems to be a stimulus for fibrogenic cell activation, so that thrombin antagonism is thought to improve e.g. progredience of liver fibrosis[25].

2.3. Innovative Thrombin Generation Tests[89]

In vivo Thrombin Activity (Sytemic 2a Activity- F2a Test)

Currently the best bio-marker for the *in vivo* thrombin generation is the test for amidolytic thrombin activity in plasma[87, 89, 90]. Thrombin entrapped in the α_2-macroglobulin cage is chromogenically determined via fast amidolysis of a tri amino acids-paranitroanilide (pNA) substrate. EDTA blood is taken and centrifuged within 1-2 h at 23°C and the resulting plasma is stabilized 1+1 with 2.5 M arginine at a pH of 8.6. In the assay 1 part of arginine-stabilized EDTA plasma can be incubated with 1 part of 0.77 mM chromogenic thrombin substrate HD-CHG-Ala-Arg-pNA in 1.25 M arginine, at a pH of 8.7 and the specific increase in absorbance (ΔA) at 405 nm with time at 37°C is determined.

In vitro Thrombin Activity

Thrombin activity determinations in plasma are extremely difficult, because ongoing plasmatic coagulation and the constant generation of other proteases such as kallikrein or fibrin (= AT-1) falsifies photometric measurements. To avoid this, the amino acid arginine is added in supra-1 molar final concentrations, which on the one side immediately stops hemostasis activation, because it competitively inhibits the active proteolytic center of the serine proteases of coagulation, and on the other side depolymerizes uncrosslinked fibrin. Based on these two properties of arginine, three ultra-specific and ultra-sensitive tests have been developed, the extrinsic coagulation activity assay (EXCA), the intrinsic coagulation activity assay (INCA), and the recalcified coagulation activity assay (RECA). All of these three new methods have in common that hemostasis activation is stopped by arginine after a certain coagulation reaction time (CRT). The chromogenic substrate is added after three minutes and the ΔA is determined. The results can be expressed in mIU (milli-international units) thrombin generated or in thrombin generation in percentage of the mean value of normal donors.

Extrinsic Coagulation Activity Assay (EXCA)

EXCA is used to measure extrinsic coagulation. 5 µl of 1 ng/ml tissue factor and 250 mM of $CaCl_2$ is added to 50 µl of plasma to activate extrinsic coagulation. After 1 min (main value) or 2 min (control value) coagulation reaction time at 37°C, 100 µl arginine-stop-reagent is added and 25 µl of 1 mM chromogenic thrombin substrate-reagent is added after 3 min. Thrombin generation is measured through photometric extinction by microtiter plate photometers with a 1 mA resolution. Plasmatic anticoagulants which influence the extrinsic or common phase of coagulation can be monitored through EXCA very accurately.

Intrinsic Coagulation Activity Assay (INCA)

INCA is used to measure intrinsic coagulation. 5 µl of 250 mM $CaCl_2$ and 5 µl of SiO_2 is added to 50 µl citrated plasma to start the coagulation reaction. After 4 min (main value) or 5 min (control value) coagulation reaction time (CRT) at 37°C, 100 µl arginine-stop-reagent is given to stop coagulation. After 3 min incubation to depolymerize uncrosslinked fibrin, 25 µl of 1 mM chromogenic thrombin substrate-reagent is added. Thrombin generation is measured through photometric extinction. Plasmatic anticoagulants which influence intrinsic or common phase of coagulation can be monitored through INCA very accurately.

Recalcified Coagulation Activity Assay (RECA)

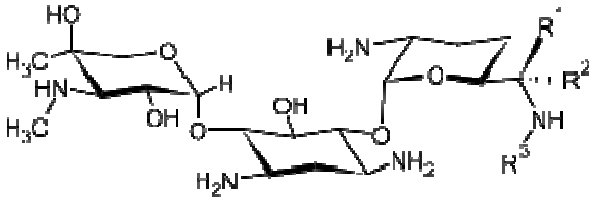
RECA is basically an ultra-sensitive INCA. 5 µl of 250 mM $CaCl_2$ is added to 50 µl of citrated plasma in pure-grade polystyrene microwells to initiate coagulation. Usually, after 20 min (main value) or 30 min (control value) at 37°C, 100 µl of arginine-stop reagent is added.

After 3 min 25 µl of 1 mM chromogenic thrombin substrate-reagent is added. Thrombin generation is measured through photometric extinction. Particularly, RECA

can be used very well to study slightest prothrombotic tendencies of plasma or changes of the plasma matrix (e.g. by drugs) in individual patients, which is its main indication.

2.4. The Aminoglycoside Gentamicin

Gentamicin is a very important aminoglycoside antibiotic[13, 34, 65]. It is produced by various species of the gram-positive bacterial genus *Micromonospora* by fermentation[45, 46, 92] as a mixture of five major related components referred to as gentamicin C1, C1a, C2, C2a, and C2b[77]. The C2-derivatives are the main type.



	R^1	R^2	R^3	Proportion
C_1	$-CH_3$	–H	$-CH_3$	20–40 %
C_{1a}	–H	–H	–H	10–30 %
C_2	$-CH_3$	–H	–H	
C_{2a}	–H	$-CH_3$	–H	40–60 %
C_{2b}	–H	–H	$-CH_3$	

Figure 2

Chemical structure of gentamicin[26]

Pure gentamicin (MW= 477.6 Daltons) consists of two amino sugar groups (to the left and to the right) and one aminociclitol group (in the middle), as illustrated in figure 2. The drug usually also contains H_2SO_4[26, 52]. It is mostly used to treat bacterial infections caused by gram-negative bacteria such as *Escherichia coli, Salmonella spp., Shigella spp., Enterobacter spp., Citrobacter spp., Acinetobacter spp., Proteus spp., Klebsiella spp., Serratia spp.,Morganella spp., Pseudomonas spp*., and by some gram-positive bacteria like *Staphylococcus aureus* and some streptococci[94]. About 50% of

gram positive staphylococci are sensible to gentamicin. The drug is not effective against *Bacteroides spp., Streptococcus pneumoniae, Burkholderia cepacia, and Strenotrophomonas maltophilia*[94]. Gentamicin is not used in the treatment of bacterial infections caused by *Neisseria gonorrhoe, Neisseria meningitides, and Legionella pneumophila* because of a feared shock reaction due to the release of lipopolysaccharides acting as pyrogenic endotoxins.

Gentamicin binds the bacterial 30S-ribosomal subunit and thus causes misreading in bacterial protein synthesis. That is why, gentamicin has no bacteriostatic, but bactericidal properties. The antibacterial effect is concentration dependent[2]. Gentamicin is given either intramuscularly, intravenously, or topically. Given orally the relatively large molecule is insufficiently absorbed from the small intestine because of its lipophobic properties[100]. Gentamicin almost does not bind to plasma proteins and does not undergo metabolisation. Gentamicin is eliminated by the kidneys with a half-life of 1.5 - 2 hours. Renal insufficiency results in a slower elimination of gentamicin and thus has an increased risk for accumulation and toxic side-effects. It is therefore important to adapt gentamicin dosage in case of renal insufficiency[2].

During prolonged treatments gentamicin can act as a vestibulotoxin[9, 78]. Approximately 5% of patients on gentamicin experience vestibular ototoxicity[74]. There seems to be a genetic predisposition to gentamicin induced vestibular dysfunction[74]; however, genetic tests are not always recommended because of the application of the drug in severe cases and critical ill patients, in which waiting for the gene analysis results could negatively influence the patient outcome[75]. Although the precise mechanisms are not still completely known, there is the supposition that gentamicin penetrates into the endolymphatic fluid of the scala media, permeates non-selective cation channels on the apical surface of hair cells, generates toxic reactive oxygen species, and furthermore interferes with other cellular pathways[48]. In detail, aminoglycosides were found to be able to block the transduction channels at the tips of stereocilia and the N-type and P/Q type channels in neurons as well as acetylcholine-evoked K^+ currents in outer hair cells. Some antioxidants such as

glutathione or other drugs such as mannitol or salicylate have been shown to protect against aminoglycosides ototoxicity in vivo[8].

A further side effect of aminoglycosides and thus gentamicin is their nephrotoxicity, which occurs in 10-20% of therapies[53]. Gentamicin is up-taken in epithelial cells of the proximal tubulus and remains there for a while, which results in nephrotoxicity[60]. Nephrotoxicity, resulting in renal dysfunction, is caused by tubular necrosis, epithelial edema of proximal tubules, cellular desquamation, tubular fibrosis, glomerular congestion, perivascular edema, and inflammation[6]. Acidic phospholipids are responsible for binding gentamicin, but more recently it has been reported that megalin, an endocytic giant receptor, is important for binding gentamicin. So megalin antagonism could be a way to prevent gentamicin-induced nephrotoxicity[60]. Nephrotoxicity clinically manifests with slow rises in serum creatinine and marked decreases in glomerular filtration rate[53].

In order to reduce side effects of a gentamicin treatment, the maximal levels should be less than 20 mg/l[39, 63]. Typical minimal plasma levels of free gentamicin are around 5 mg/l, typical maximal plasma levels are around 10 mg/l.

Currently there are some studies about gentamicin dosing; whether once-daily giving is more effective than several times giving and which plasma level should be reached in different bacterial infections. These studies mostly concern pediatric gentamicin dosing[27, 31, 37, 39, 43, 100]. The reason for these studies is the so called first exposure effect; if the bacterial cell survives the first exposure to gentamicin, a subsequent second exposure will not be effective as the first. This is the reason why, gentamicin is more effective given once in a high dosage than given several times in small doses[2].

2.5. The disseminated intravascular coagulation

Pathologic disseminated intravascular coagulation (PIC) is a very complex and dangerous condition[1, 5, 33, 35, 37, 38, 49, 64, 67, 96, 97]. It is caused by enhanced systemic intravascular coagulation activation on the bottom of various underlying conditions[47] like shock, intravascular haemolysis, release of tissue factor, bacterial endotoxins, proteolytic enzymes, particulate, or colloidal matter anoxia and anoxaemia, endothelial damage and ingestion of certain lipid substances[54]. This is followed by massive systemic generation of thrombin. Low levels of antithrombin (AT-3) insufficiently inhibit coagulation activation[30]. The result is circulating micro-thrombi, ischemia, and organ failure. Seldomly, massive consumption coagulopathy, so-called overt DIC, can occur with massive bleeding plus intravasal fibrin clotting. Early diagnosis and early treatment are essential for the outcome of the patient. Systemic thrombin activity is an important biomarker in the phase of pre-PIC[90] and antithrombin, fibrinogen, platelets, and prothrombin time seem to be important hemostatic markers for the begin of the overt DIC phase[99].

2.6. Background of the work

Coagulation activation and thus activation of thrombin play a very important role in clinical daily work. There are many diseases or deaths caused by systemic or local micro- or macro-thrombi, pathological activations of the coagulation system.

In such situations it would be important to know if drugs could additionally trigger the patient coagulation system and aggravate his condition. Some drugs are known triggers for pathological coagulation activation (e.g. asparaginase) and there is the suspicion that the important aminoglycoside gentamicin can trigger coagulation activation, too. Because gentamicin is often used in treatment of sepsis and sepsis is a possible cause of pathologic disseminated intravascular coagulation, it would be important to know if gentamicin itself could further aggravate the already affected coagulation of these patients. The question is:

Does gentamicin influence plasmatic thrombin generation?

3. Plasma, Materials and Methods

3.1. Materials

3.1.1. Equipment

Mikrotiter plate photometer

Milenia; DPC, Los Angeles, USA

Digitally controlled water – bath (37°C)

Memmert; Büchenbach, Germany

Sterile 37°C incubator

Melag; Berlin, Germany

3.1.2. Material

Polypropylene monovettes containing 0.5 ml 106 mM sodium citrate

Sarstedt; Nümbrecht, Germany

Polystyrene U-wells (highest quality)

Brand; Wertheim, Germany; article nr. 701300

Multipette

Eppendorf; Hamburg, Germany

3.1.3. Chemicals

Gentamicin

Gentamicin-ratiopharm®40SF, Ulm, Germany;

The drug vial contained 1 ml 67.8 mg gentamicin · H_2SO_4 = 40 mg gentamicin;

1 mg gentamicin sulphate = 590 µg gentamicin.

Enoxaparin

Clexane®; Aventis; Frankfurt, Germany

$CaCl_2$, arginine, Triton X 100®

Sigma; Deisenhofen, Germany

Siliconized 20 ml glass vials

Siemens-DadeBehring; Marburg, Germany; article nr. OVKE49

Chromogenic thrombin substrate

HD-CHG-Ala-Arg-pNA

Pentapharm; Basel, Switzerland; 100 mg article nr. 081-03

Purified bovine thrombin (130 IU/vial)

Siemens-DadeBehring

Human albumin

CSL Behring; Marburg, Germany

0.9% NaCl

Braun Melsungen, Germany

3.1.4. Plasma samples

This study has been approved by the local ethic commission of the university hospital of Marburg (181/09). N=139 unfrozen individual normal platelet poor citrated plasma from healthy volunteers who gave written informed consent and N=11 pools of unfrozen normal plasma, consisting of 5-20 individual samples, were analysed. The blood samples derived from voluntary students either from the laboratory or during the practical course of Clinical Chemistry. Before drawing blood and after written consent, the donors were asked if they had a coagulation disease or if they were on anticoagulation treatment. The study was performed only with normal samples. Normal plasma was considered as fresh, if the time between withdrawal and test was less than or equal to 2h. More than 2h to 6h old plasma was considered as normal. The citrated blood was centrifuged at 2800 g and 23°C for 10 minutes. The cell poor plasma was used for the study.

3.2. Methods

3.2.1. The recalcified coagulation activity assay (RECA)

50 µl of individual normal platelet poor citrated plasma (0.5-6 h 23°C old) and pooled normal plasma, consisting of 5-20 individual samples, was supplemented with 0-19.6 mg/l (final plasma concentration) gentamicin by immediate repetitive 1+1 dilution in high-quality polystyrene U-wells microtiter plates. Additionally, 5 samples of individual unfrozen normal plasma were added 50 µl of gentamicin· H_2SO_4 as well as 0 IU/ml or 0.5 IU/ml of enoxaparin-natrium by an Eppendorf-multipette® in order to analyse the prophylactic action of this low molecular weight heparin on gentamicin induced thrombin generation. Because of its ultra-specificity and ultra-sensitivity to detect slightest prothrombotic tendencies of plasma, the recalcified coagulation activity assay (RECA) was performed immediately thereafter.

RECA can be sub-divided in two phases: firstly the thrombin generation phase and secondly the thrombin detection phase.

1. Thrombin generation phase

In the first phase 5 µl of RECA-trigger is added in duplicate by an Eppendorf-multipette® with 0.9% NaCl-rinsed in new disposable polypropylene-tips and the plates are shaken strongly for five seconds. The RECA-trigger consists of 250 mM of $CaCl_2$ out of 4 ml frozen/thawed aliquots in siliconized glass. After performing coagulation reaction times of 0-30 minutes at a temperature of 37°C, the coagulation reaction is stopped by 100 µl of arginine-stop-reagent. Concerning the samples with a coagulation reaction time of 0 minutes, the arginine-stop reagent is added previous to the RECA trigger.

2. Thrombin detection phase

In the second phase the plasmatic turbidity is measured with a microtiter plate photometer with a 1 mA resolution at 405 nm at room temperature of 23°C after 3 minutes. For this purpose 25 µl aliquoted frozen/thawed 1 mM chromogenic thrombin substrate HD-CHG-Ala-Arg-pNA is added in 1.25 M of arginine (CS) with a pH of 8.7. Afterwards the extinction $\Delta A_{405nm}/t$ is measured at a temperature of 37°C.

The RECA was standardised with 0.1 IU/ml of purified bovine thrombin in 6 % human albumin. This substituted the plasma sample and produced a specific extinction $\Delta A_{405nm}/t$ of about 3 mA/min at a temperature of 37°C. The maximal extinction ΔA_{405nm} (complete cleavage of the chromogenic substrate) was about 1000 mA; 40 % of maximum = 400 mA (= upper limit of linear range). The thrombin generation was designated as the thrombin activity generated during the coagulation reaction time minus the basal plasmatic thrombin activity at 0 minutes coagulation reaction time (basal F2a in normal citrated plasma = 8$\pm$2 mIU/ml; MV$\pm$1SD). The approximate 200 % stimulatory concentration was determined in the ascending part of the time vs. thrombin generation curve. Only thrombin activities being in the ascending part of the curve (the ratio between F2a activity at time point 1 divided by F2a activity at time point 2 has to be less than 1) are of relevance, because there is nearly no generated nascent fibrin (= antithrombin-1) that can entrap thrombin and inhibit its further generation.

4. Results

4.1. Thrombin generation by gentamicin in individual fresh normal plasma

4.1.1. Thrombin generation by gentamicin in 18 individual fresh normal plasmas

After written consent, 18 blood samples deriving from healthy voluntary students were drawn and shortly afterwards centrifuged. 50 µl from every platelet-poor plasma was pipetted with a gentamicin concentration of 0-19.6 mg/l by repetitive 1+1 dilution in polystyrene u-wells microtiter plates. Afterwards RECA was performed with 5, 10, 15, 20, 25, and 30 minutes coagulation reaction time and RECA-20 was found out to be the incubation time, during which thrombin is typically generated.

Because fresh plasma is the most significant, the results of these 18 plasma samples are very relevant for the question if gentamicin can trigger thrombin generation. These 18 samples were measured about 30 minutes after being drawn. Plasma samples kept for hours before being analysed may be pre-activated and have a higher basal activity of activated clotting factors generated by surface contact activation in blood tubes during *in vitro* time [98].

The figures 4a-c illustrate the thrombin generation of these 18 samples after 20 minutes coagulation reaction time. The 18 thrombin generation curves are illustrated in 3 figures respectively with 6 curves for a better overview. The tested plasmas are continuously labelled with N1, N2, N3, NX; N standing for normal plasma.

The thrombin activity range between 0.01-0.1 IU/ml is the most significant, because there is enough generated thrombin and the generated thrombin activity is not too high. Too high activities would produce significant amounts of fibrin, which, as antithrombin-1, would absorb thrombin and interfere with its activity [57] and by this cause artefacts in thrombin measurements. These artefacts would be graphically represented as sudden drops of the thrombin generation curve after the relevant ascending part. Therefore the x-axis with the gentamicin concentration stops in

certain graphs at values smaller than 19.6 mg/l. This was done for a clearer presentation of the ascending part of the thrombin generation curve and because the relevant question of the present work is, which gentamicin concentration is needed to generate twofold thrombin and thus the approximate 200% stimulatory concentration, which can be read in the early ascending part of the graph in most cases. The y-axis varies respectively to the generated thrombin activity. According to results there are strong responders that react to a very low or low dosage of gentamicin between 0.1-2.5 mg/l with twofold enhanced thrombin generation, moderate responders, with twofold increased thrombin generation at gentamicin concentrations between 2.5-6 mg/l, low responders that react to a medium or high dosage of gentamicin between 6-19.6 mg/l with twofold enhanced thrombin generation, and resistant responders, whose thrombin generation is not influenced by gentamicin concentrations of smaller than or equal to 19.6 mg/l.

RESPONDER	**APPROX. 200% GENTAMICIN STIMULATORY CONCENTRATION in mg/l**
Strong responder	0.1-2.5
Moderate responder	2.5-6
Low responder	6 -19.6
Resistant responder	>19.6

Figure 3

Classification criteria for the approx. 200% stimulatory concentrations of the responders

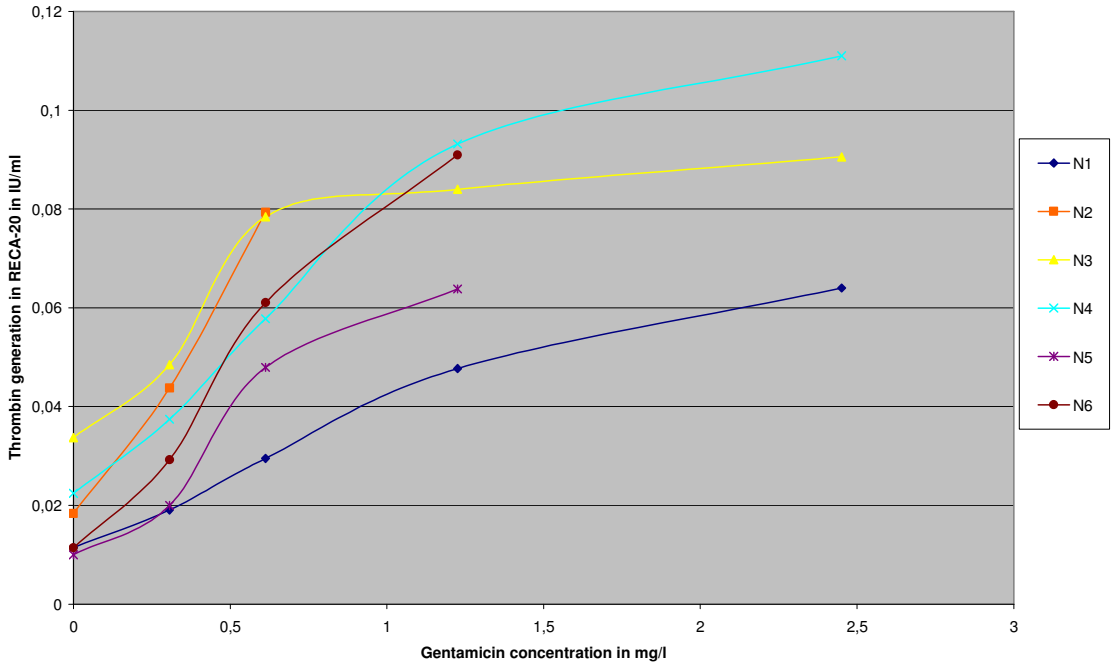


Figure 4a

Thrombin generation in the individual fresh normal plasmas N1-N6 expressed in IU/ml

Figure 4a illustrates the thrombin generation in 6 of 18 fresh normal plasmas after gentamicin supplementation. The normal plasmas N1-N6 are examples for very strong responders to gentamicin addition. They already react to very low triggering gentamicin dosages of smaller than 0.5 mg/l with twofold enhanced thrombin generation, as illustrated in the ascending part of their curves. Plasma N6 even doubles its thrombin generation at a very low gentamicin concentration of 0.2 mg/l gentamicin. Its graph is steep from the beginning; it has a basal thrombin activity of 0.01 IU/m and reacts to 0.2 mg/l of gentamicin with a thrombin activity of 0.02 IU/ml, as shown in the N6 graph. These results are very representative and relevant, because they demonstrate the gentamicin ability of triggering thrombin generation and they show that very low approximate 200% stimulatory concentrations can already trigger

thrombin generation. Additionally, the analysed plasmas were fresh (about 0.5 h at room temperature) and thus of high significance.

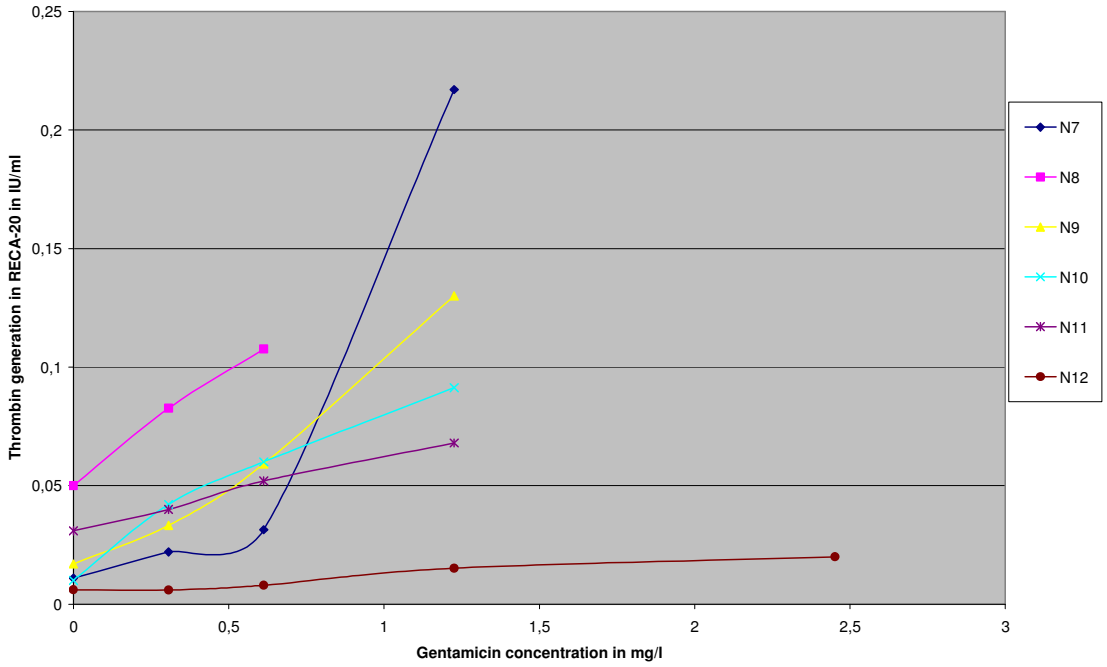


Figure 4b

Thrombin generation in the individual fresh normal plasmas N7-N12 expressed in IU/ml

Figure 4b illustrates the thrombin generation in 6 of 18 fresh normal plasmas after gentamicin supplementation. The normal plasmas N7-N12 are also strong responders to gentamicin addition, reacting to gentamicin dosages between 0.3-1 mg/l with twofold increased thrombin generation. N10 is the strongest responder. Its curve starts at a basal thrombin activity of 0.01 IU/ml and reaches a thrombin activity of 0.02 IU/ml at a gentamicin concentration of 0.2 mg/l.

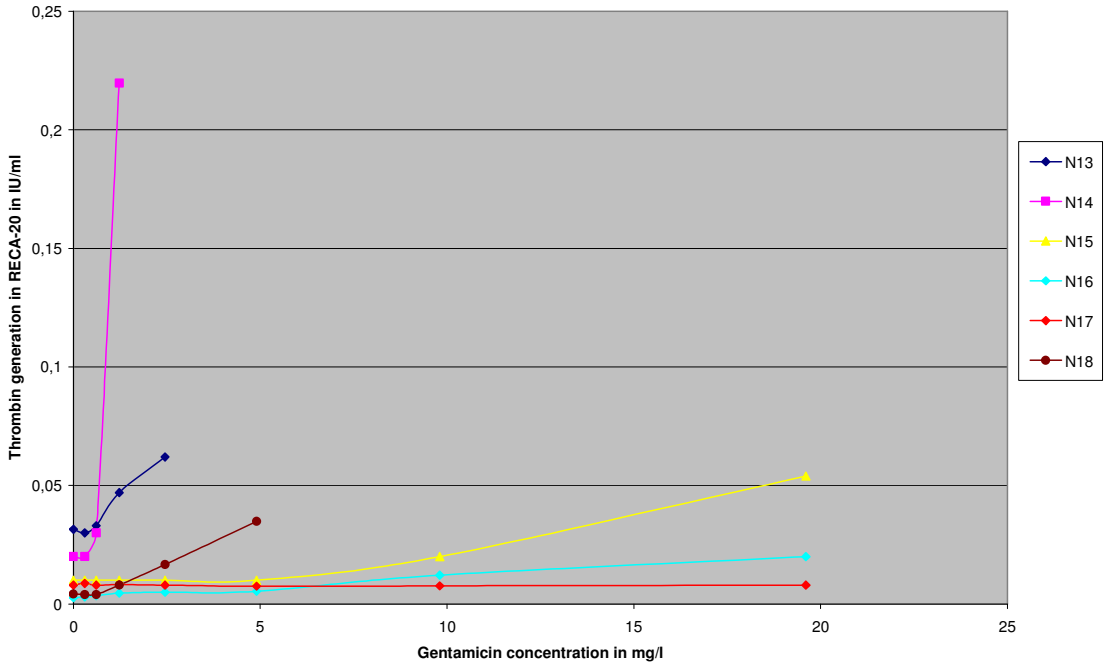


Figure 4c

Thrombin generation in the individual fresh normal plasmas N13-N18 expressed in IU/ml

Figure 4c illustrates the thrombin generation in 6 of 18 fresh normal plasmas after gentamicin supplementation. The normal plasmas N13, N14 and N18 can be interpreted as strong responders; their triggering dosage is between 1-2 mg/l of gentamicin. The normal plasma N15 is a low responder reacting to a dosage of 10 mg/l of gentamicin with twofold thrombin generation, whereas N16 is a moderate responder with an approx. 200% stimulatory concentration of 5 mg/l of gentamicin. The normal plasma N17 is an example for a resistant responder. Its thrombin generation is not influenced by gentamicin concentrations up to 19.6 mg/l.

15 plasmas out of these 18 samples of fresh normal plasma are strong responders. Their thrombin generation is doubled by gentamicin dosages between 0.2-2 mg/l. One plasma is a moderate responder. Its thrombin generation is twofold increased by

a gentamicin dosage of 5 mg/l. One plasma is a low responder with an approx. 200% stimulatory concentration of 10 mg/l of gentamicin. One plasma is found to be resistant. The following table shows the approx. 200% stimulatory concentrations of these 18 samples sorted by test persons. The mean value for the 200% approx. stimulatory concentration is 1.4 mg/l of gentamicin, the standard deviation is 2.5.

Normal Plasma	**Approx. SC200 [mg/l]**
N1	0.35
N2	0.25
N3	0.5
N4	0.35
N5	0.3
N6	0.2
N7	0.3
N8	0.6
N9	0.3
N10	0.2
N11	1
N12	1
N13	2
N14	1
N15	10
N16	5
N17	resistant
N18	1
MV*	1.4
SD*	2.5

*Resistant responders are not considered

Figure 5

Approx. 200% stimulatory concentrations of 18 individual fresh normal plasmas

A pool was mixed out of these 18 individual fresh normal plasmas and 50 µl of it was pipetted in polystyrene u-wells microtiter plates and supplemented with 0-19.6 mg/l of gentamicin by repetitive 1+1 dilution. Afterwards, RECA with 5, 10, 15, 20, 25, and 30 minutes coagulation reaction time was performed with the observation of pooled

fresh normal plasma needing a shorter coagulation reaction time than individual plasma probably because of the ability for one susceptible plasma to trigger the whole pool. After being activated, the susceptible plasma activates the other plasmas, leading to a significant thrombin generation. This is probably also the reason for the approx. 200% stimulatory concentrations of pooled plasma being lower compared to individual plasma. The pooled plasma of these 18 samples shows a significant thrombin generation at a coagulation reaction time of 15 minutes. Pools are continuously designated with P1, P2, etc. The following figure illustrates pool P1 mixed out of the 18 samples of fresh plasma.

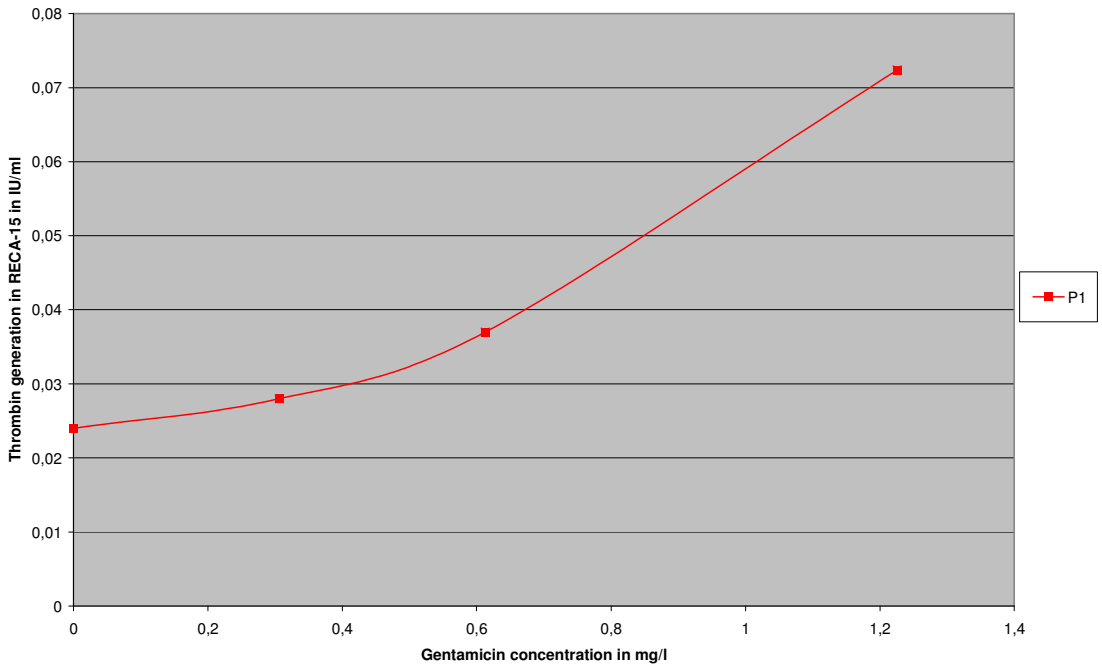


Figure 6

Thrombin generation in the pooled fresh normal plasma P1 expressed in IU/ml

The red thrombin generation curve starts at a higher basal thrombin activity probably caused by the plasma samples activating each other. The thrombin generation is twofold enhanced at a gentamicin concentration of 0.8 mg/l. This approx. 200%

stimulatory concentration is lower than the mean value of individual measurements probably because one sensible plasma can activate and influence the others in their thrombin generation leading to twofold enhanced thrombin generation at lower gentamicin concentrations.

4.1.2. Thrombin generation by gentamicin in 14 individual fresh normal plasmas

Other 14 blood samples of voluntary donors were drawn during their practical course of Clinical Chemistry after written consent. After centrifugation 50 µl of every individual fresh platelet-poor plasma was pipetted with a gentamicin concentration of 0-19.6 mg/l by repetitive 1+1 dilution in polystyrene u-wells microtiter plates. Afterwards RECA with 5, 10, 15, 20, 25, and 30 minutes coagulation reaction time was performed. Once again, 20 minutes was confirmed to be the most effective incubation time for fresh plasma. Since the blood samples were kept only 2 h at room temperature before being processed, these results are also very significant. Due to a clearer presentation, the results are subdivided in 2 figures, figure 7a and 7b, respectively with 7 curves. Once again, attention must be paid on the activity range between 0.01-0.1 IU/ml, being the most significant range.

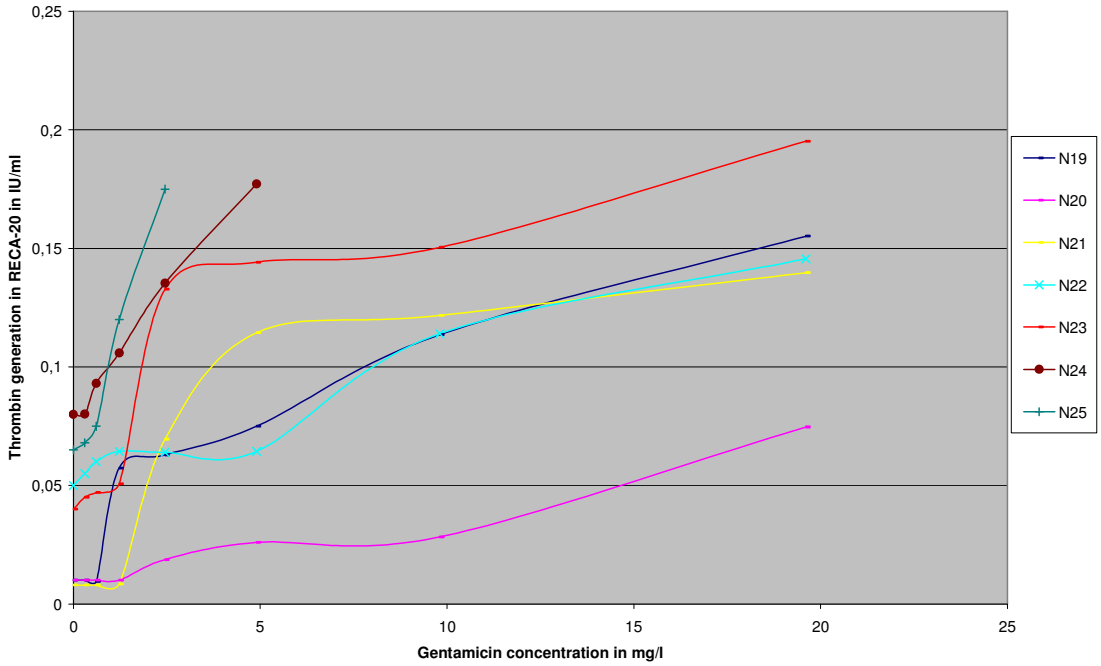


Figure 7a

Thrombin generation in the individual fresh normal plasmas N19-N25 expressed in IU/ml

Figure 7a illustrates the thrombin generation in 7 of 14 fresh normal plasmas after gentamicin supplementation. The normal plasmas N19, N21, N23, and N25 are strong responders to gentamicin addition. These plasmas react to the low triggering gentamicin dosages between 0.8-2 mg/l with a twofold enhanced thrombin generation, as shown in the ascending part of their curves. The normal plasmas N20 and N24 are moderate responders. Their approx. 200% stimulatory concentrations are between 3.2-4 mg/l of gentamicin. The normal plasma N22 is a low responder: it doubles its thrombin generation at a concentration of 8 mg/l of gentamicin.

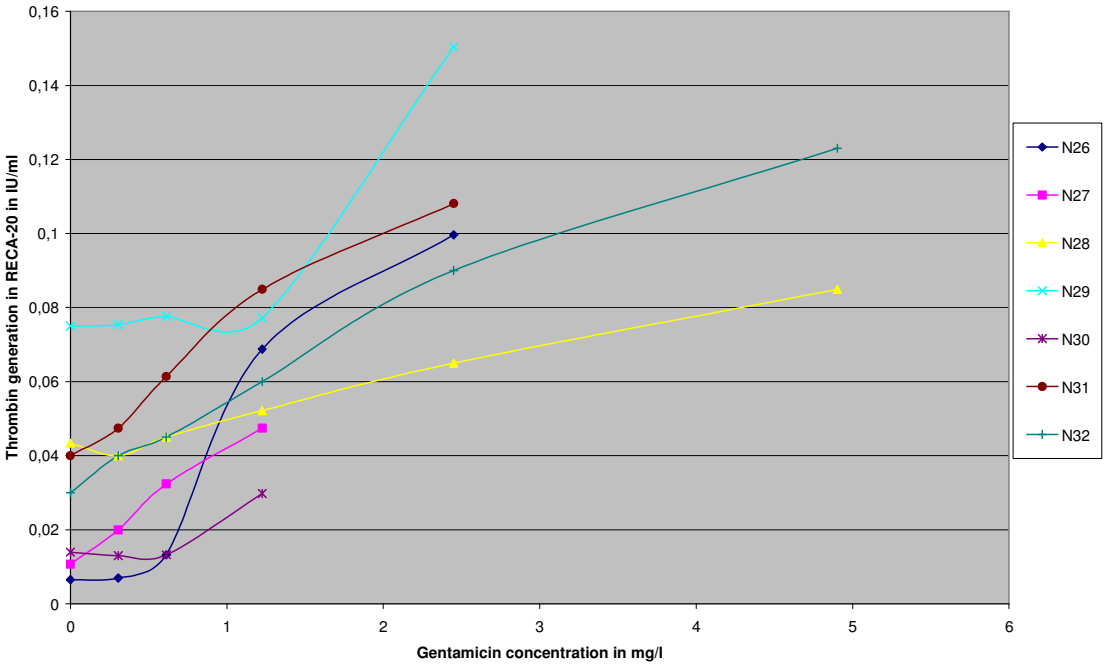


Figure 7b

Thrombin generation in the individual fresh normal plasmas N26-32 expressed in IU/ml

Figure 7b shows the thrombin generation in 7 of 14 fresh normal plasmas after gentamicin supplementation. The normal plasmas N26, N27, N29, N30, N31, and N32 are examples for strong responders. Their approx. 200% stimulatory concentrations are between 0.3-2.4 mg/l of gentamicin. The strongest responder of this group is N27. Its thrombin generation curve starts at a basal activity of 0.01 IU/ml. It enhances twofold its thrombin generation at a gentamicin concentration of 0.3 mg/l, as shown at the second point of its curve. The plasma sample N28 can be interpreted as a moderate responder. Its thrombin generation curve is quite flat; its approx. 200% stimulatory concentration is 4.8 mg/l.

10 out of these 14 samples of fresh normal plasma are strong responders. Their thrombin generation is doubled by gentamicin dosages of 0.3-2.4 mg/l. 3 plasmas are moderate responders. Their thrombin generation is twofold enhanced by gentamicin dosages between 3.2-4.8 mg/l. One plasma was a low responder; it doubles its

thrombin generation at a gentamicin dosage of 8 mg/l of gentamicin. The following table shows the approx. 200% stimulatory concentrations of these 14 samples sorted by test person. The mean value for the 200% approx. stimulatory concentrations is 2.3 mg/l of gentamicin, the standard deviation is 2.1.

Normal Plasma	Approx. SC200 [mg/l]
N19	0.8
N20	3.2
N21	2
N22	8
N23	1.6
N24	4
N25	1.6
N26	0.6
N27	0.3
N28	4.8
N29	2.4
N30	1.1
N31	1.2
N32	1.2
MV	2.3
SD	2.1

Figure 8

Approx. 200% stimulatory concentrations of 14 individual fresh normal plasmas

The approximate 200% stimulatory concentrations of these 14 samples are slightly higher than the approx. 200% stimulatory concentrations of the 18 samples presented in chapter 4.1.1 previously. Most of the 18 samples had very low triggering gentamicin dosages, whereas these 14 samples show higher approx. 200% stimulatory concentrations. The explanation for this difference is probably the 18 samples being kept only 0.5 h at room temperature before examination. The probability of coagulation activation by self activation and contact activation with the blood tubes is increased by the time it takes to start with RECA. If coagulation is basically activated,

contact factors are consumed and more contact trigger (gentamicin) is needed to achieve the approx. 200% stimulatory concentration.

The pool P2 was mixed out of these 14 individual fresh normal plasmas. 50 µl of P2 was pipetted in polystyrene u-wells microtiter plates and supplemented with 0-19.6 mg/l of gentamicin by repetitive 1+1 dilution. Afterwards, RECA with 5, 10, 15, 20, 25, and 30 minutes coagulation reaction time was performed. Once again, pooled plasma needed a shorter coagulation reaction time than individual plasma. RECA-15 was the best incubation time with a significant thrombin generation. The reason for this is e.g. a strong responder, reacting to low triggering gentamicin dosages and activating the other plasmas. For the same reason the approx. 200% stimulatory concentration of pooled plasma is lower than the calculated mean value of the approx. 200% stimulatory concentrations of the 14 individual samples.

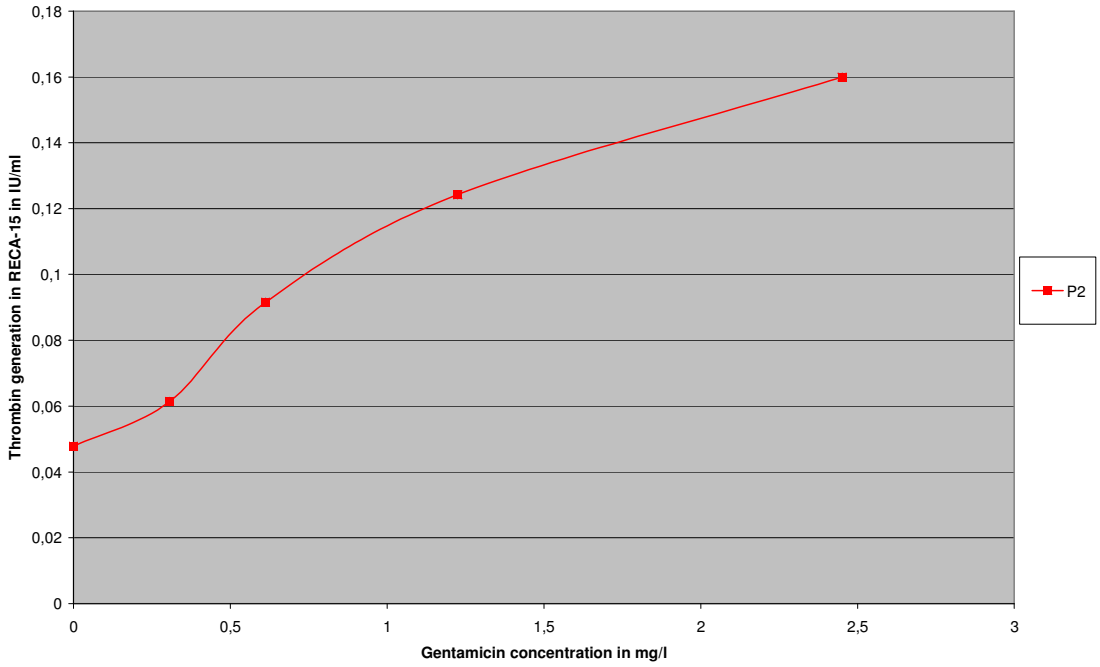


Figure 9

Thrombin generation in the pooled fresh normal plasma P2 expressed in IU/ml

The thrombin generation curve starts at a basal thrombin activity of ca. 0.05 IU/ml, higher than in individual normal plasma, probably because the plasma samples activated each other. Then, the curve ascends and reaches a thrombin activity of 0.1 IU/ml at a gentamicin concentration of 0.6 mg/l, which is the approx. 200% stimulatory concentration. Once again, this low value indicates the ability for one susceptible plasma to trigger the whole pool. Pooled plasma seems to behave similarly to normal plasma as far as gentamicin triggered thrombin generation is concerned.

These 32 samples, the 18 of chapter 4.1.1 and the 14 of chapter 4.1.2, were the freshest and thus the most significant samples which were analysed. They were kept only 0.5-2 h at room temperature before examination. They answer the question if gentamicin can trigger thrombin generation with a clear “yes”: 25 of 32 normal plasmas doubled their thrombin generation at gentamicin dosages between 0.2-2.4 mg/l. 10 of 32 plasmas even reacted to very low gentamicin dosages smaller or equal than 0.5 mg/l with twofold increased thrombin generation.

In the next chapters it is shown how normal plasma, kept 2-6 h at room temperature, behaves after gentamicin supplementation. Then, some examples of plasmas are illustrated which do not have a stimulatory concentration, but an inhibitory concentration. At last, the question is answered if the pathologic thrombin generation can be prevented by the low molecular weight heparin enoxaparin-natrium (Clexane®).

4.2. Thrombin generation in individual normal plasma

2-6 h old normal samples deriving from healthy voluntary students were analysed as well. Shortly after centrifugation 50 µl of every platelet-poor plasma was pipetted with a gentamicin concentration of 0-19.6 mg/l by repetitive 1+1 dilution in polystyrene u-wells microtiter plates. Afterwards RECA with 5, 10, 15, 20, 25, and 30 minutes coagulation reaction time was performed. Normal plasma was observed to have a different incubation time than fresh normal plasma. An incubation time of 10 to 15 minutes was sufficient to generate thrombin. This is probably a consequence of the plasma being older and so pre-activated either through auto contact activation or through the contact to blood tube surface. If the selected incubation time was too short, the approx. SC200 resulted too high or the plasma appeared to be resistant to gentamicin. The decisive criterion was shown to be a sufficient basal thrombin activity. If there was no basal activity, the approx. 200% stimulatory concentrations could not be measured and the incubation time had to be extended to 15 or 20 minutes. The normal plasma N33, illustrated in figure 10, is an example for RECA-10 being a too short incubation time. The time has to be extended to 15 minutes to measure a significant thrombin generation. The normal plasma N34, shown in figure 11, illustrates the impossibility to determine the approx. 200% stimulatory concentration if there is no basal thrombin activity. In this case the incubation time has to be extended as well.

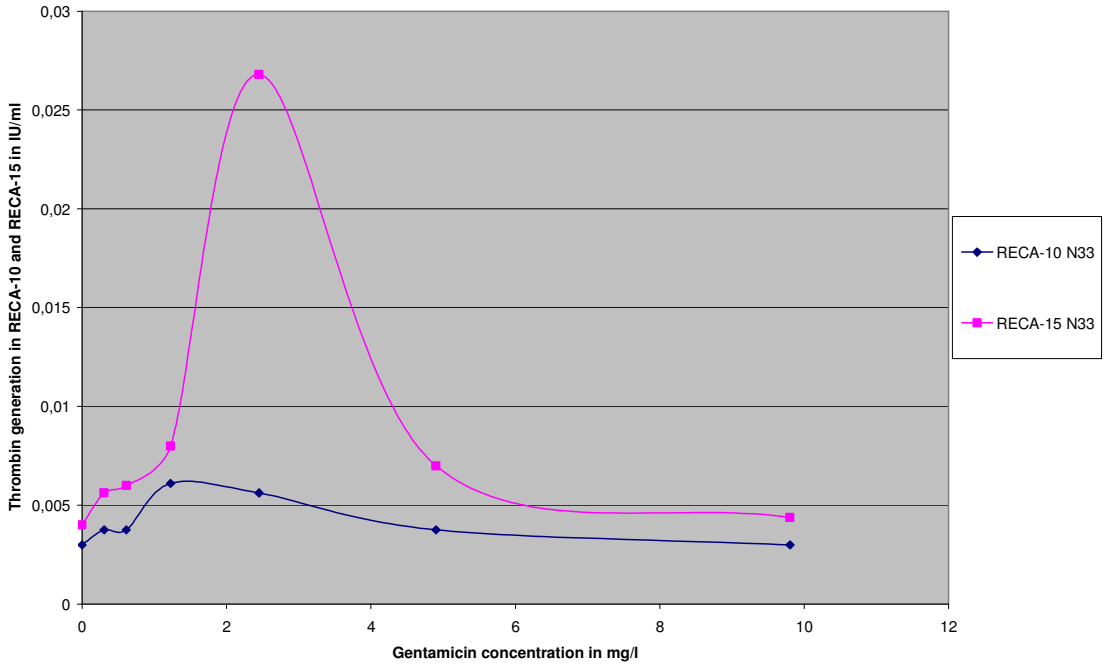


Figure 10

Thrombin generation in the individual normal plasma N33 expressed in IU/ml

Figure 10 demonstrates the thrombin generation in the normal plasma N33 after gentamicin supplementation. The thrombin generation curve of RECA-10 shows the insufficiency of 10 minutes incubation time. The curve does not develop appropriately and is quite flat. The incubation time has to be extended to RECA-15 in order to generate a more significant thrombin generation curve. As designated from RECA-15, the approx. 200 % stimulatory concentration is 1.2 mg/l of gentamicin. The thrombin generation curve of RECA-15 shows a further interesting phenomenon: after a strong thrombin generation (ascending part of the curve) thrombin gets entrapped in fibrin (=antithrombin 1). The thrombin generation is stopped; the curve suddenly drops (descending part of the curve).

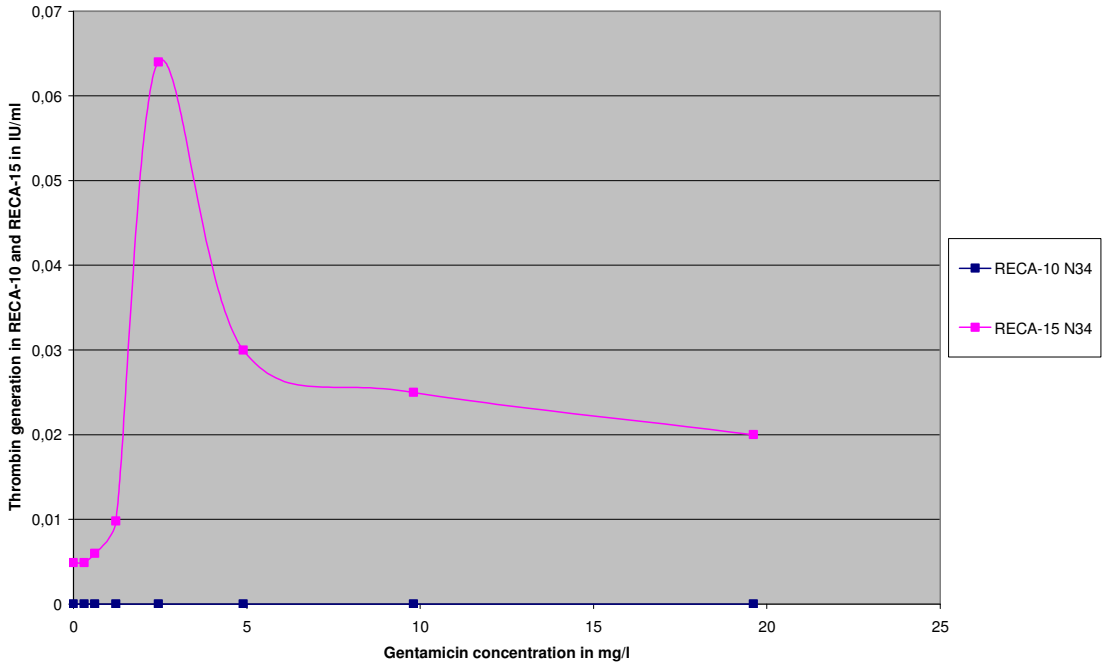


Figure 11

Thrombin generation in the individual normal plasma N34 expressed in IU/ml

Figure 11 illustrates the thrombin generation in the normal plasma N34 after gentamicin supplementation. The normal plasma N34 shows no basal thrombin activity in the RECA-10 thrombin generation curve. There is no significant thrombin generation and the approx. 200% stimulatory concentration can not be estimated. After extending the incubation time to 15 minutes, the normal plasma N34 shows a significant thrombin generation. As determined from RECA-15, the approx. 200% stimulatory concentration is 1.2 mg/l of gentamicin.

4.2.1. Thrombin generation by gentamicin in 14 individual normal plasmas

The figures beneath illustrate the thrombin generation by gentamicin in 14 individual normal plasmas deriving from healthy voluntary students. For a better overview the 14 graphs of thrombin generation are represented in four figures, figure 12a, 12b, 12c and 12d, respectively with 3, 4, 3 and 4 curves. The normal plasmas continue to be designated with N35, N36 etc. After arriving at the laboratory and being centrifuged, 50 µl of each platelet-poor plasma was pipetted with a gentamicin concentration of 0-19.6 mg/l by repetitive 1+1 dilution in polystyrene u-wells microtiter plates. Afterwards RECA with 5, 10, 15, 20, 25 and 30 minutes coagulation reaction time was performed. The best incubation time with a significant thrombin generation was found to be RECA-15. Once again, the thrombin activity range between 0.01-0.1 IU/ml is the most significant. There is enough generated thrombin and the generated thrombin activity is not too high, so fibrin can not interfere with thrombin activity.

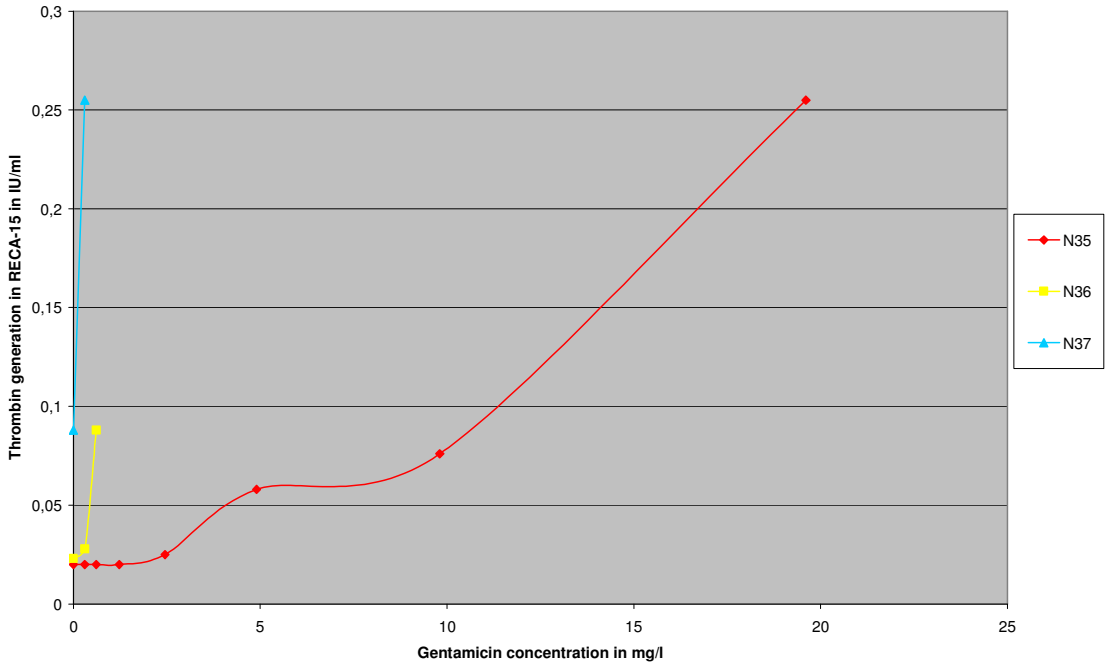


Figure 12a

Thrombin generation in the individual normal plasmas N35-N37 expressed in IU/ml

Figure 12a illustrates the thrombin generation in the normal plasmas N35-N37 after gentamicin supplementation. The normal plasmas N36 and N37 are very strong responders. They react to gentamicin addition with a twofold enhanced thrombin generation at gentamicin dosages of respectively 0.5 and even 0.1 mg/l, whereas the normal plasma N35 is a moderate responder. Its approx. 200% stimulatory concentration is of 4 mg/l of gentamicin.

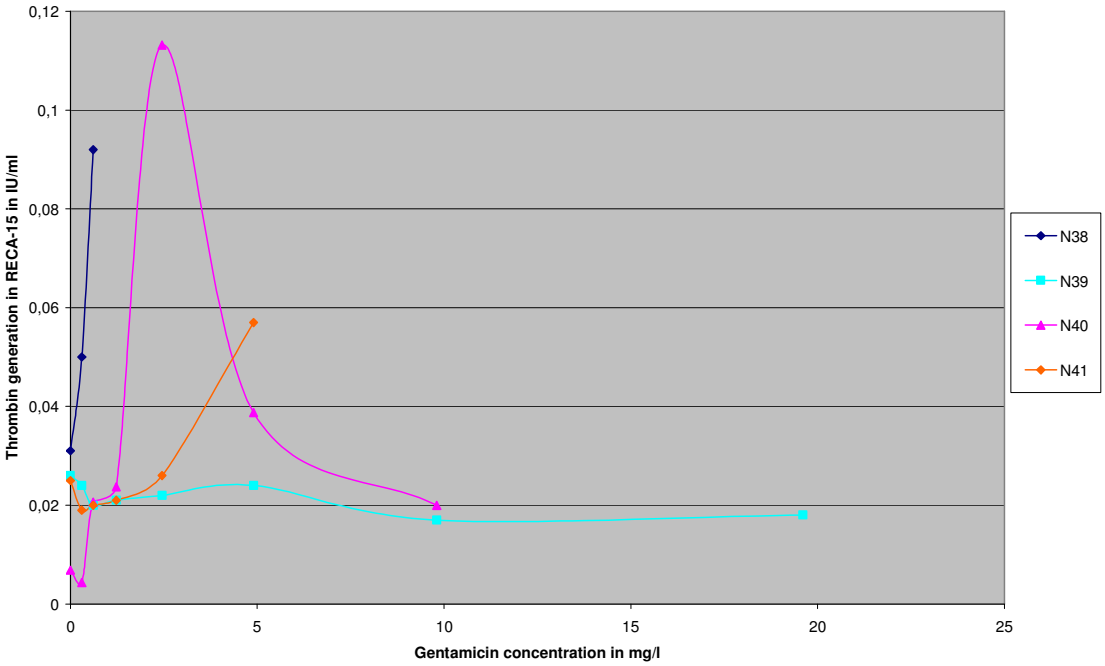


Figure 12b

Thrombin generation in the individual normal plasmas N38-N41 expressed in IU/ml

Figure 12b shows the thrombin generation in the normal plasmas N38-N41 after gentamicin supplementation. The plasma sample N38 is a further example for a very strong responder. Its basal thrombin activity starts at 0.03 IU/ml and doubles at a gentamicin concentration of 0.3 mg/l. The plasma N39 can be interpreted as resistant. Its thrombin generation curve is constantly flat after gentamicin addition, apart from little non significant increases and decreases. The plasma N40 is a further very strong responder with an approx. 200% stimulatory concentration of 0.5 mg/l of gentamicin. The normal plasma N41 is a moderate responder. Its thrombin activity doubles at a gentamicin concentration of 4 mg/l, as shown in the ascending part of its graph.

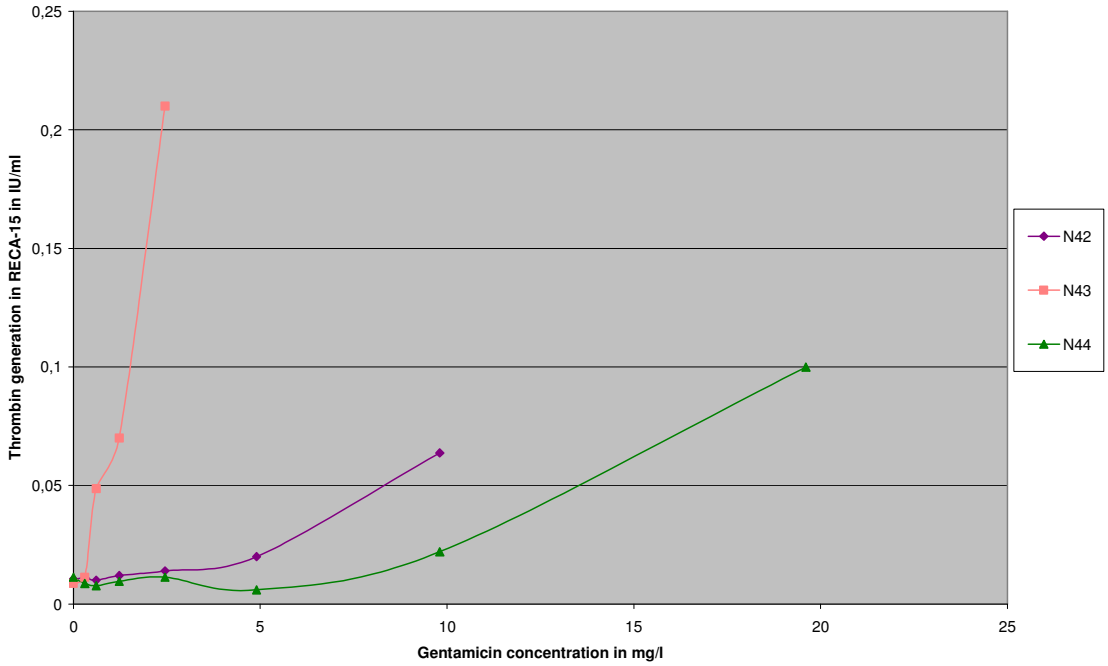


Figure 12c

Thrombin generation in the individual normal plasmas N42-N44 expressed in IU/ml

Figure 12c represents the thrombin generation in the normal plasmas N42-N44 after gentamicin supplementation. N42 is a moderate responder. As illustrated in its purple graph, its basal thrombin activity starts at 0.01 IU/ml and is doubled at a gentamicin concentration of 5 mg/l. The normal plasma N43 is a very strong responder with a steep thrombin generation curve. Its approx. 200% stimulatory concentration is 0.5 mg/l. The plasma N44 is a low responder: its approx. 200% stimulatory concentration is 10 mg/l of gentamicin.

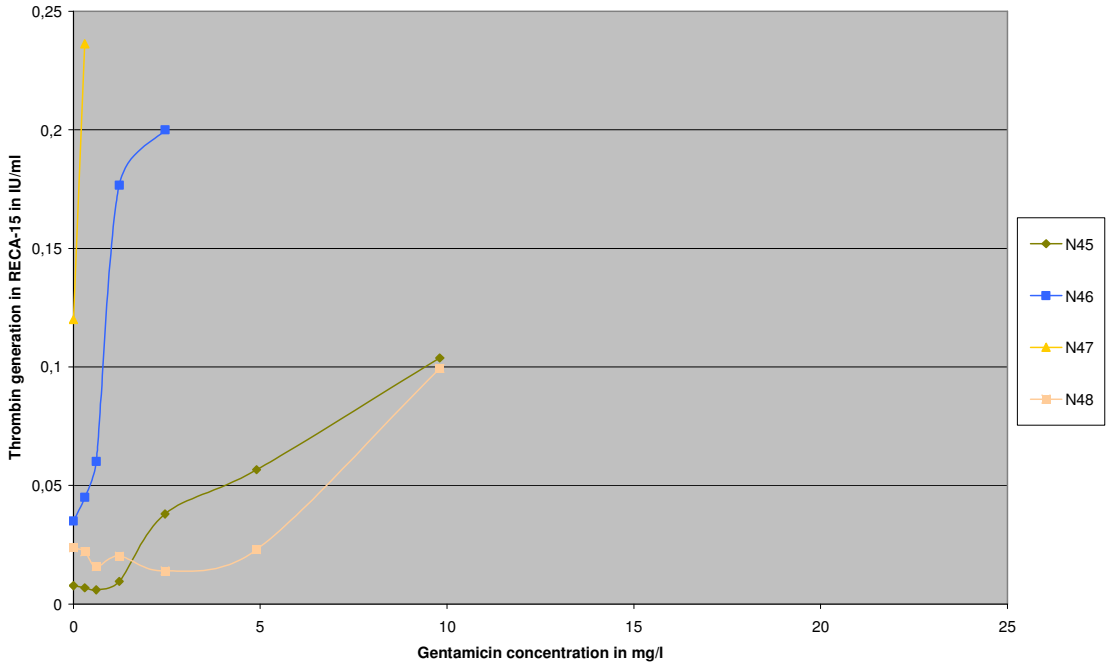


Figure 12d

Thrombin generation in the individual normal plasmas N45-N48 expressed in IU/ml

Figure 12d illustrates the thrombin generation in the normal plasmas N45-N48 after gentamicin supplementation. Plasma samples N45, N46, and N47 are strong responders with approx. 200% stimulatory concentrations between 0.3-1.5 mg/l. N48 is a low responder. Its thrombin generation curve starts to ascend at a gentamicin concentration of 5 mg/l; at a concentration of 7 mg/l its thrombin generation is doubled.

8 out of these 14 samples of normal plasma are strong responders. Their thrombin generation is doubled by gentamicin dosages between 0.1-1.5 mg/l. Three plasmas are moderate responders. Their thrombin generation is twofold enhanced by gentamicin concentrations of 4-5 mg/l. Two plasmas are low responders: they double their thrombin generation at gentamicin dosages between 7-10 mg/l. One plasma is resistant. The following table shows the approx. 200% stimulatory concentrations of

these 14 samples sorted by test person. The mean value of the approx. 200% stimulatory concentrations is 2.4 mg/l of gentamicin, the standard deviation is 3.1.

Normal Plasma	Approx. SC200 [mg/l]
N35	4
N36	0.5
N37	0.1
N38	0.3
N39	resistant
N40	0.5
N41	4
N42	5
N43	0.5
N44	10
N45	1.5
N46	0.7
N47	0.3
N48	7
MV*	2.4
SD*	3.1

*Resistant reponders are not considered

Figure 13

Approx. 200% stimulatory concentrations of 14 individual normal plasmas

The pool P3 was mixed out of these 14 individual normal plasmas and 50 µl of it was pipetted in polystyrene u-wells microtiter plates and supplemented with 0-19.6 mg/l of gentamicin by repetitive 1+1 dilution. Afterwards, RECA with 5, 10, 15, 20, 25, and 30 minutes coagulation reaction time was performed. Interestingly, there was a significant thrombin generation at RECA-10 and RECA-20, RECA-20 being the better incubation time of the both and thus longer than in fresh plasma. Previously, the fresh normal pools P1 and P2 showed how pooled plasma needed a shorter incubation time than individual plasma. This difference is probably caused by 10 minutes being a too short incubation time for these 14 unfrozen samples. Possibly, some samples did not

have a sufficient basal thrombin activity yet or the most susceptible sample was not activated, so the incubation time had to be extended.

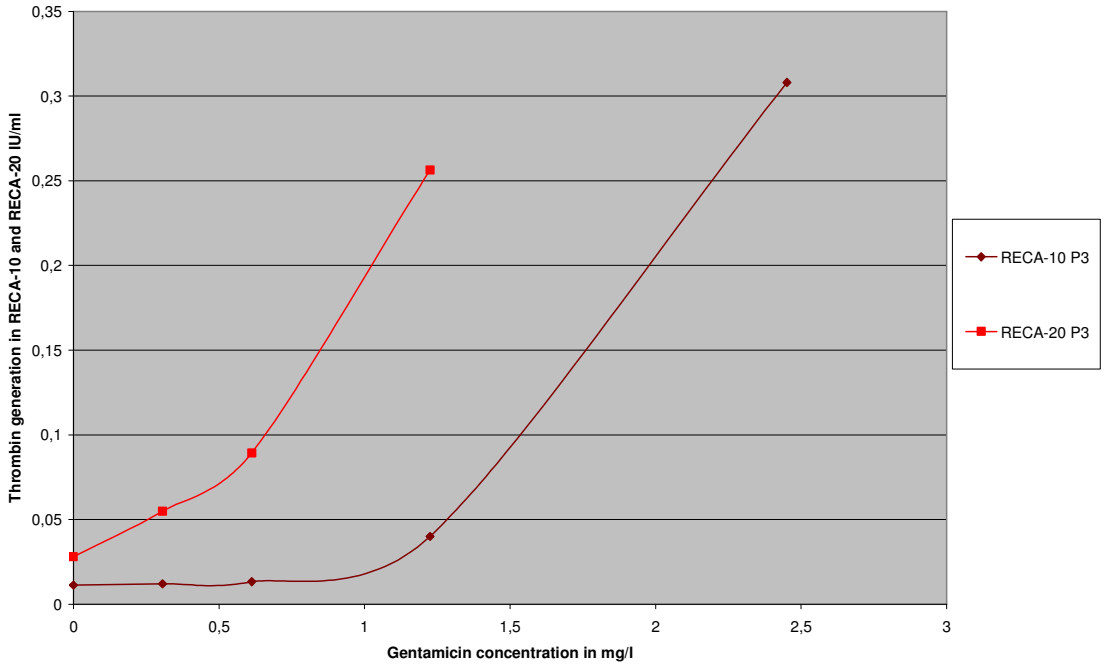


Figure 14

Thrombin generation in the pooled normal plasma P3 expressed in IU/ml

Figure 14 illustrates the thrombin generation in the pooled normal plasma P3 mixed out of 14 individual normal plasmas. The thrombin generation curve of 10 minutes incubation time starts at a basal thrombin activity of 0.01 IU/ml. It proceeds quite flat until coagulation is activated. Its approx. 200% stimulatory concentration is 1.1 mg/l. In contrast to the RECA-10 curve, the RECA-20 curve starts at a basal thrombin activity of 0.025 IU/ml; the graph is steeper and thrombin generation is twofold enhanced at a gentamicin concentration of 0.3 mg/l. Because of the different approx. 200% stimulatory concentrations of the two graphs and the quite high approx. 200% stimulatory concentration of 1.1 mg/l in RECA-10, the RECA-20 graph with its approx. 200% stimulatory concentration of 0.3 mg/l is probably the relevant one. Previous measurements of thrombin generation in pooled plasma showed how the

incubation time of pooled plasma was lower than in individual plasma, like the pools P1 and P2 illustrated.

In conclusion, normal plasma behaves similarly to fresh normal plasma as far as gentamicin triggered thrombin generation is concerned. Once again, gentamicin led to coagulation activation and thus to thrombin generation. 7 of 14 samples reacted to very low gentamicin dosages between 0.1-0.7 mg/l with twofold thrombin generation.

During measurements some individual normal plasma samples did not have an approx. 200% stimulatory concentration, but they had an inhibitory concentration. Thrombin generation was not enhanced by gentamicin, but initially inhibited. The following figures, figure 15 and figure 16, illustrate two examples of normal plasma, N49 and N50, with such an inhibitory concentration.

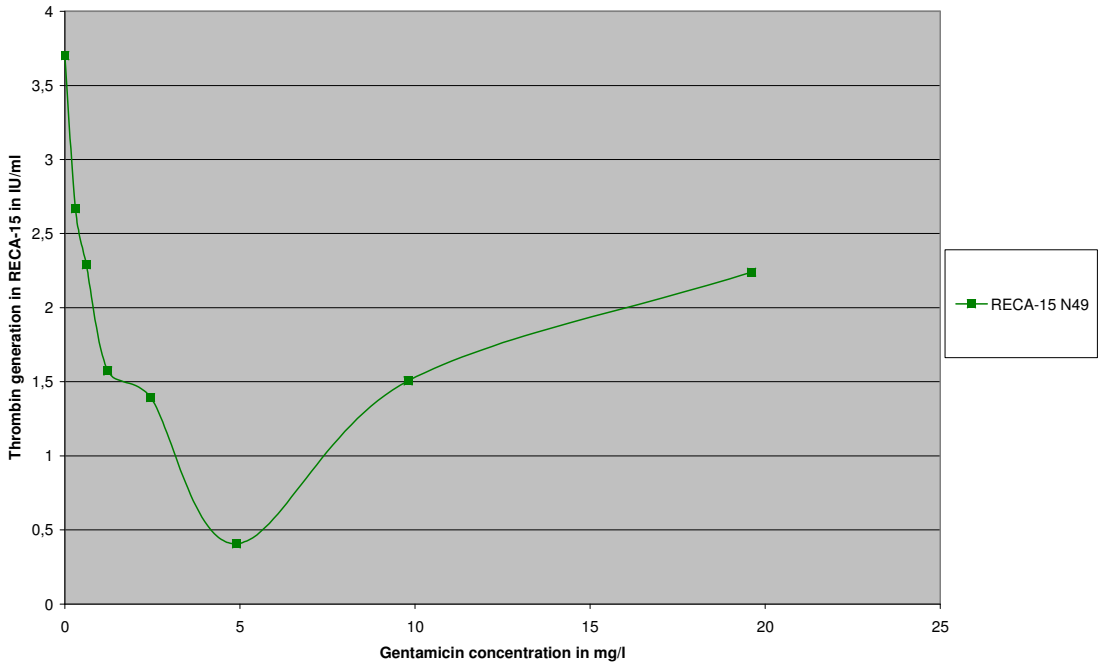


Figure 15

Thrombin generation in the individual normal plasma N49 expressed in IU/ml

Figure 15 illustrates the thrombin generation in the individual normal plasma N49 after gentamicin supplementation. Interestingly, the basal thrombin activity is high with 3.7 IU/ml. After gentamicin addition there is not an enhancement in thrombin generation but the curve decreases. At a gentamicin concentration of 1 mg/l the thrombin activity decreases to 1.85 IU/ml. This value was designated as the approx. 50% inhibitory concentration, the gentamicin concentration used to reduce the thrombin activity by half. The graph starts to ascend at a gentamicin concentration of 5 mg/l, but does not overtake or even reach the initial thrombin activity of 3.7 IU/ml.

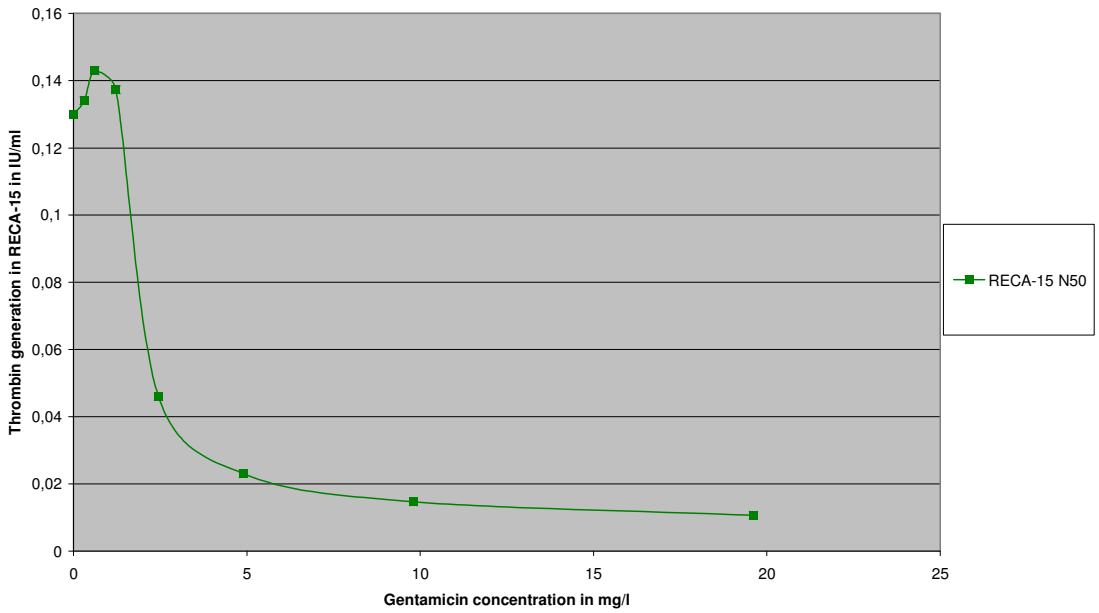


Figure 16

Thrombin generation in the individual normal plasma N50 expressed in IU/ml

Figure 16 illustrates the thrombin generation in the individual normal plasma N50 after gentamicin supplementation. The graph starts at a basal thrombin activity of 0.13 IU/ml. After gentamicin addition the thrombin activity initially decreases very strong, after reaching the gentamicin concentration of 5 mg/l, it falls slower. The thrombin activity is reduced by half at a gentamicin concentration of 2.5 mg/l, which is the approx. 50% inhibitory concentration.

These approx. 50% inhibitory concentrations found in 3 of 139 examined plasmas are more of theoretical interest. To determine if the inhibitory concentration is an analytical artefact or not, the coagulation assays INCA (intrinsic coagulation activity assay) or EXCA (extrinsic coagulation activity assay) have to be performed. They systematically measure intrinsic and extrinsic coagulation factors, and can prove if gentamicin can also inhibit or inactivate coagulation factors, and thus inhibit thrombin generation.

4.3. Prevention of gentamicin induced thrombin generation by the low molecular weight heparin Enoxaparin-Natrium (Clexane®)

Because of the observed gentamicin effect on thrombin generation, we asked the question if a low molecular weight heparin can prevent this pathologic thrombin generation. For this purpose, enoxaparin-natrium (Clexane®) was chosen, because this low molecular weight heparin is currently the clinical standard for prophylaxis and therapy of thromboembolic affections. Enoxaparin-natrium mainly inactivates factor 10a, a very important coagulation factor of both the intrinsic and extrinsic pathway of coagulation and thus inhibits thrombin generation. A therapy with enoxaparin can be monitored by measuring the anti-factor 10a activity. Many authors see the anti-factor 10a activity between 0.1-0.4 IU/ml as the prophylactic range, whereas the 0.5-1.1 IU/ml activity range is used for therapeutic indications[103]. It needs to be considered that 0.1-0.4 IU/ml is probably a too low dosage for critical ill patients, who run a greater risk of thromboembolism. Critical ill patients, treated with gentamicin, may present hypercoagulability or have pre-activated plasmas because of the systemic inflammatory response to a bacterial infection. In this case, a higher prophylactic dosage of enoxaparin-natrium may be more appropriate. In 2013 Robinson et al. analysed whether 1mg/kg of enoxaparin daily was a more effective dosage for thromboprophylaxis in critical ill patients than the standard dosage of 20-40 mg daily. The study was based on the priori assumption that critical ill patients may need a higher anti-factor 10a activity range than 0.1-0.4 IU/ml, because of their higher risk of thromboembolism[73]. A dosage of 0.5 IU/ml was therefore used for prophylaxis and designated as the ideal dosage for prevention of gentamicin induced thrombin generation. The following four figures, figure 17a, 17b, 17c, and 17d, show four examples of individual normal plasmas, N51, N52, N53, and N54, in which the gentamicin induced thrombin generation is prevented by administration of 0.5 IU/ml enoxaparin-natrium (Clexane®). The samples were kept at room temperature for 2-6 hours. After arriving at the laboratory and being centrifuged, 50 µl samples of every platelet-poor plasma was pipetted with a gentamicin concentration of 0-19.6 mg/l by repetitive 1+1 dilution in polystyrene u-wells microtiter plates.

Additionally, 50 µl of every platelet-poor plasma was pipetted with a gentamicin concentration of 0-19.6 mg/l by repetitive 1+1 dilution and 0.5 IU/ml of enoxaparin-natrium (Clexane®) in polystyrene u-wells microtiter plates. Then, thrombin generation was measured in RECA-15 for both. The graphs illustrate the gentamicin induced thrombin generation with and without Clexane® addition.

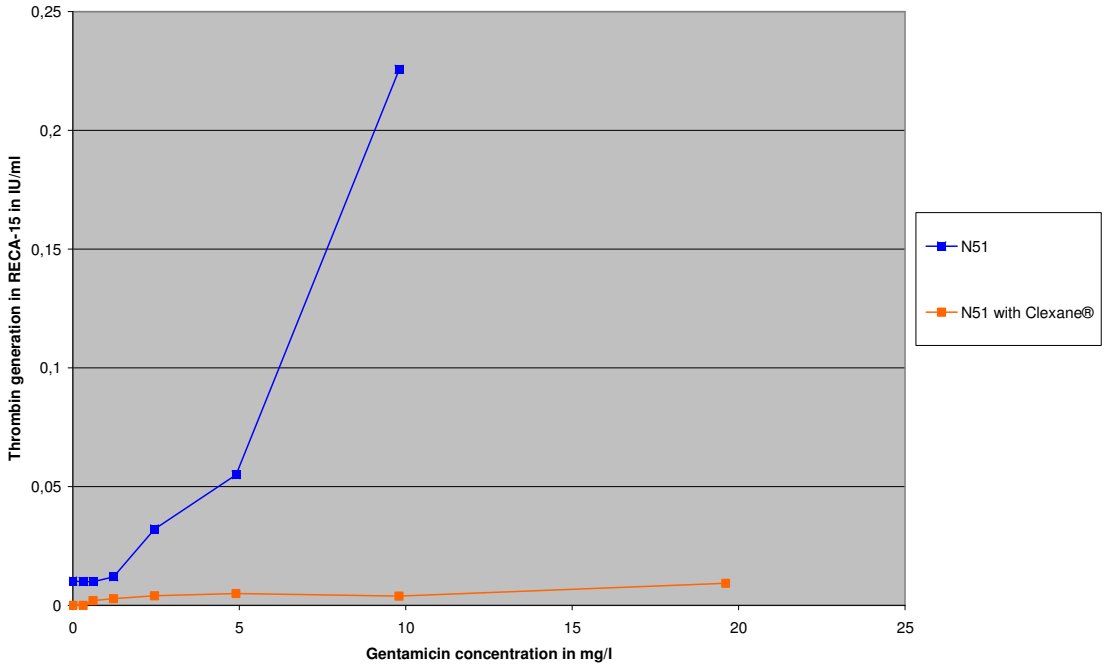


Figure 17a

Prophylaxis of gentamicin induced thrombin generation by 0.5 IU/ml Clexane®

Figure 17a illustrates the thrombin generation in the individual normal plasma N51 after gentamicin supplementation (blue curve) and after gentamicin supplementation with additionally 0.5 IU/ml enoxaparin-natrium administration (orange curve). The blue curve starts at a basal thrombin activity of 0.01 IU/ml, it ascends steeply and constantly and doubles its thrombin generation at a gentamicin concentration of 2 mg/l. The same plasma behaves completely different when both enoxaparin and gentamicin are added. The basal thrombin activity is low at 0.006 IU/ml, the thrombin

generation curve remains flat permanently, and there is no relevant thrombin generation.

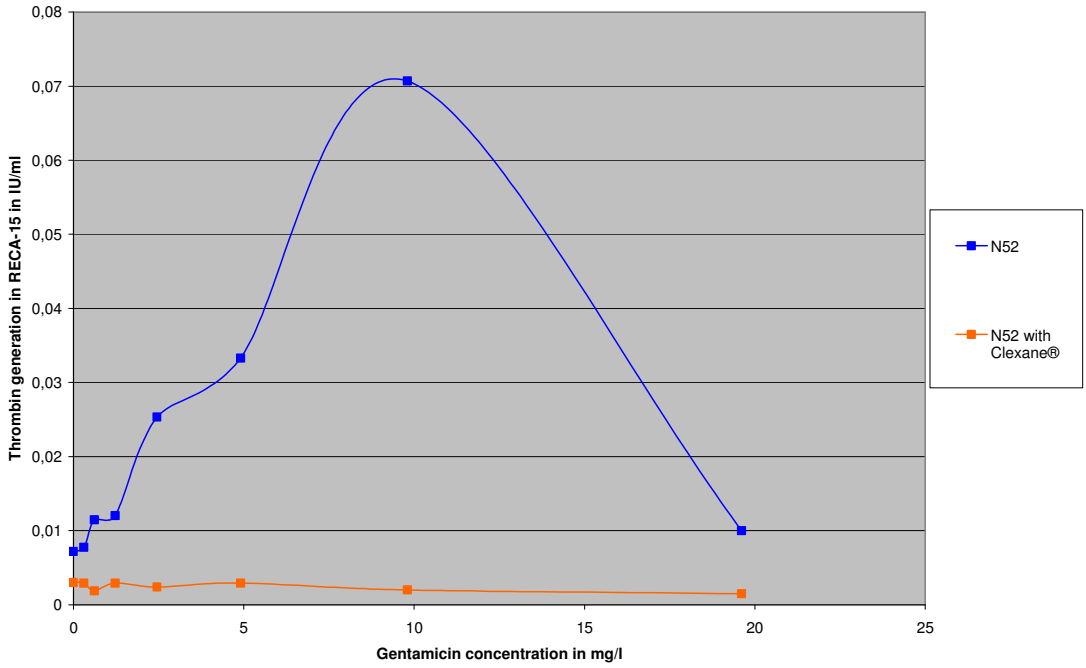


Figure 17b

Prophylaxis of gentamicin induced thrombin generation by 0.5 IU/ml Clexane®

Figure 17b shows the thrombin generation in the individual normal plasma N52 after gentamicin supplementation (blue curve) and after gentamicin supplementation with additionally 0.5 IU/ml of enoxaparin-natrium administration (orange curve). The blue curve starts at a basal thrombin activity of 0.007 IU/ml. After gentamicin addition the thrombin generation curve ascends, its approx. 200% stimulatory concentration is 2 mg/l of gentamicin. The orange curve has an initial thrombin activity of 0.003 IU/ml. After Clexane® and gentamicin are added to the normal plasma, there is no relevant thrombin generation. The plasma seems to be resistant to gentamicin.

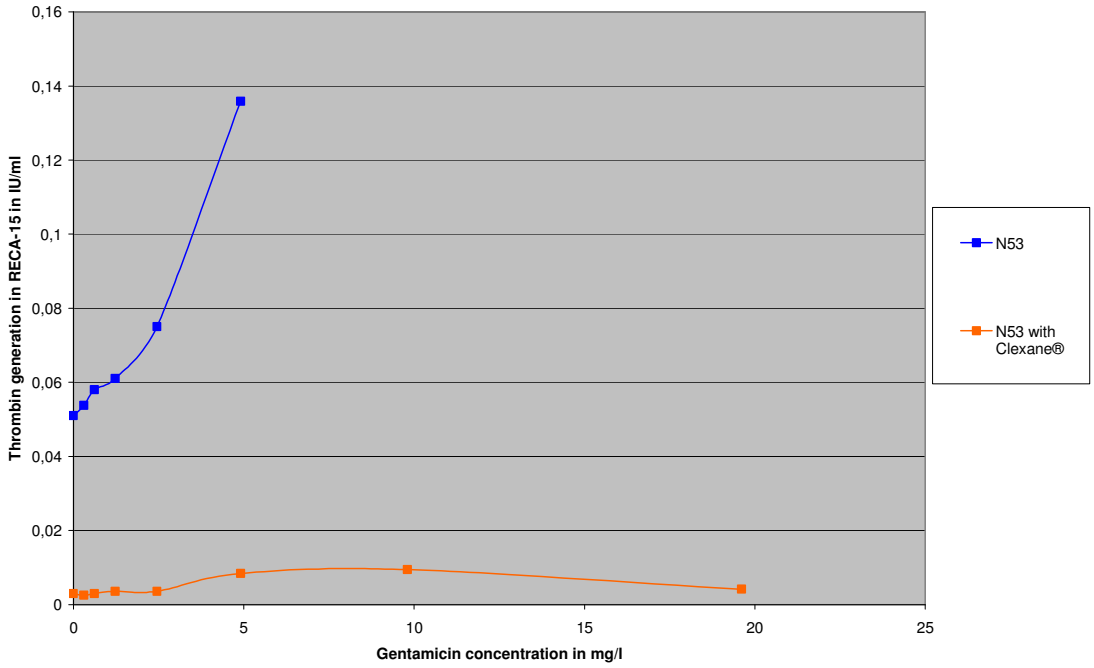


Figure 17c

Prophylaxis of gentamicin induced thrombin generation by 0.5 IU/ml Clexane®

Figure 17c demonstrates the thrombin generation in the individual normal plasma N53 after gentamicin supplementation (blue curve) and after gentamicin supplementation with additionally 0.5 IU/ml enoxaparin-natrium administration (orange curve). Once again, there is a clear difference between the two graphs: The blue curve of normal plasma N53 starts at a basal thrombin activity of 0.05 IU/ml and ascends after gentamicin addition. Its thrombin generation increases; at a gentamicin concentration of 4 mg/l its thrombin generation is twofold enhanced. If enoxaparin-natrium is given additionally with gentamicin to its plasma, the graph behaves completely different, as illustrated in the orange graph. There is only little thrombin generation and it does not reach values higher than 0.01 IU/ml.

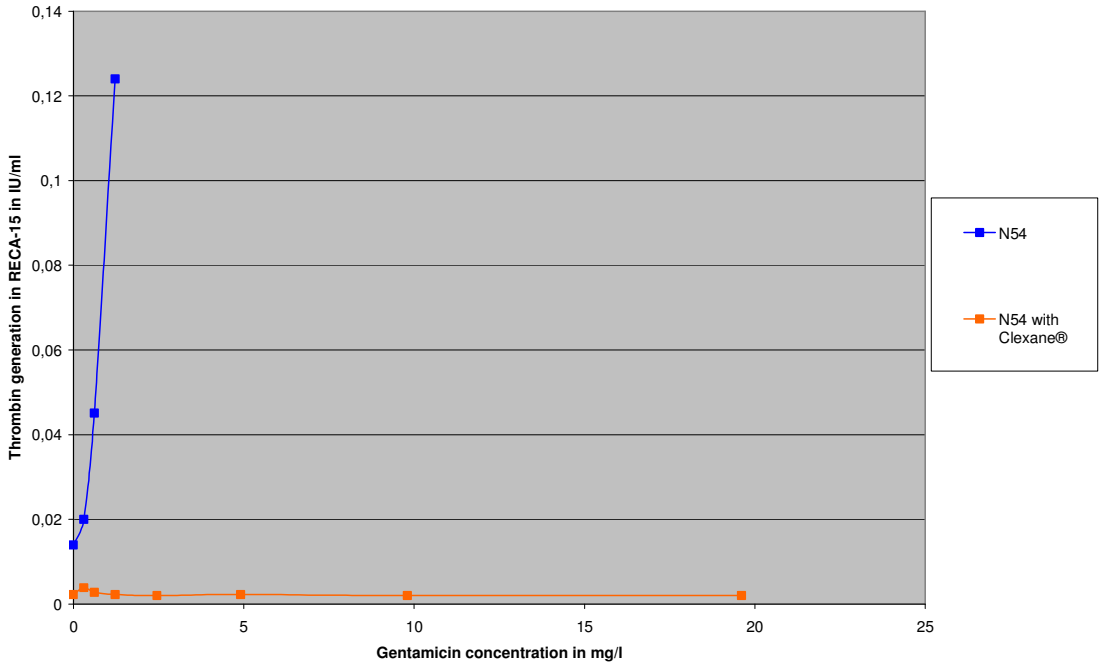


Figure 17d

Prophylaxis of gentamicin induced thrombin generation by 0.5 IU/ml Clexane®

Figure 17d demonstrates the thrombin generation in the individual normal plasma N54 after gentamicin supplementation (blue curve) and after gentamicin supplementation with additionally 0.5 IU/ml enoxaparin-natrium administration (orange curve). After gentamicin addition the normal plasma N54 behaves like a very strong responder, as shown in the blue graph. The curve is steep; its thrombin generation is doubled at a gentamicin concentration of 0.5 mg/l. The plasma appears to be resistant to gentamicin, when Clexane® is additionally given. There is no significant thrombin generation; the thrombin generation curve is constantly flat.

Concluding, 0.5 IU/ml of enoxaparin-natrium prevented the gentamicin induced thrombin generation. When Clexane® was given additionally with gentamicin to the normal plasmas, they never had a thrombin generation of higher than 0.1 IU/ml, although the same plasmas reacted with an increased or strong thrombin generation when only gentamicin was added to their plasmas.

4.4. Conclusion

The presented results showed how gentamicin triggers thrombin generation. It was illustrated how normal plasma reacts to gentamicin with an enhanced thrombin generation after 15-20 minutes of incubation time. The generated thrombin was measured with the recalcified coagulation activity assay and the gentamicin susceptibility appeared to be individually different. There were strong responders, moderate responders, low responders, and resistant responders. 36% (50 out of 139) of the analysed plasma samples showed an approx. SC200 of < 1 mg/l of gentamicin, 12% (16 out of 139) even had an approx. SC200 of < 0.5 mg/l of gentamicin. The pathologic gentamicin induced thrombin generation could be prevented by 0.5 IU/ml of the low molecular weight heparin enoxaparin-natrium (Clexane®). The following figure, figure 18, shows the distribution of the approx. 200% stimulatory concentrations of the 139 analysed individual normal plasmas.

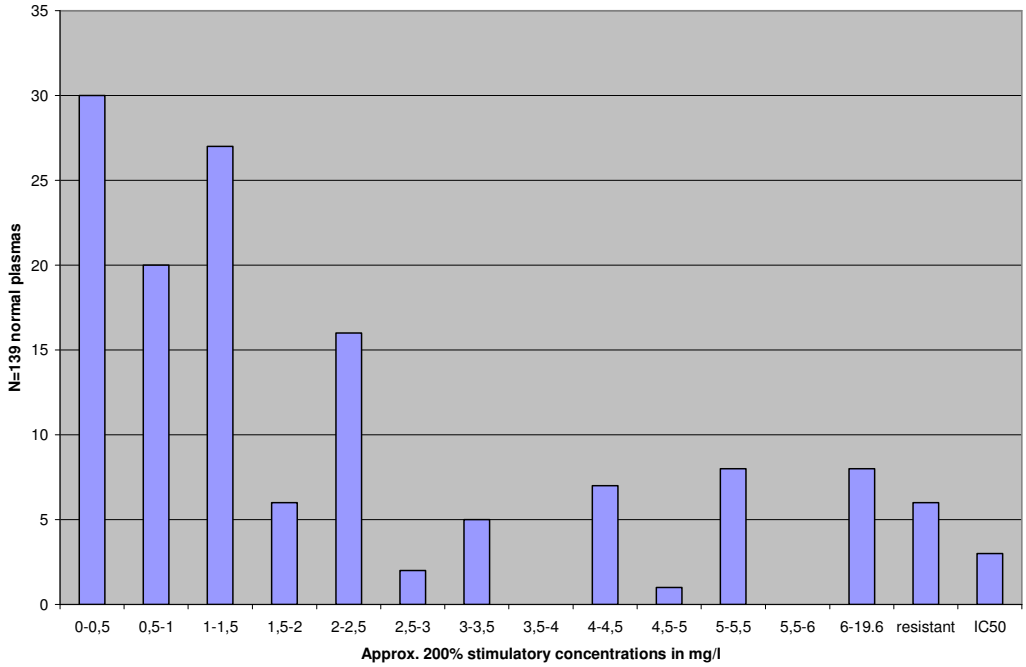


Figure 18

Distribution of the approx. 200% stimulatory concentrations in 139 normal plasmas

Figure 18 illustrates the distribution of the approx. 200% stimulatory concentrations of the 139 analysed individual normal plasmas. 77 out of 139 distribute around 0.1-1.5 mg/l gentamicin.

All the approx. 200% stimulatory concentrations of the 139 analysed individual normal plasmas are sorted by test person and listed in the following table. The illustrated approx. 200 stimulatory concentrations were determined at 15-20 minutes coagulation reaction time. The mean value is 2, the standard deviation is 2.5.

Normal Plasma	**Approx. 200% SC**
N1	0.35
N2	0.25
N3	0.5
N4	0.35
N5	0.3
N6	0.2
N7	0.3
N8	0.6
N9	0.3
N10	0.2
N11	1
N12	1
N13	2
N14	1
N15	10
N16	5
N17	resistant
N18	1
N19	0.8
N20	3.2
N21	2
N22	8
N23	1.6
N24	4
N25	1.6
N26	0.6
N27	0.3
N28	4.8
N29	2.4
N30	1.1
N31	1.2
N32	1.2
N33	1.2
N34	1.2
N35	4

N36	0.5
N37	0.1
N38	0.3
N39	resistant
N40	0.5
N41	4
N42	5
N43	0.5
N44	10
N45	1.5
N46	0.7
N47	0.3
N48	7
N49	IC50 1
N50	IC50 2.5
N51	2
N52	2
N53	4
N54	0.5
N55	1
N56	1
N57	2
N58	2
N59	2
N60	1
N61	1
N62	1
N63	3
N64	0.3
N65	1
N66	5
N67	1.5
N68	1
N69	6
N70	1
N71	resistant
N72	3
N73	0.5
N74	2
N75	2.5
N76	13
N77	1
N78	3
N79	3
N80	1

N81	0.5
N82	1.5
N83	0.4
N84	1
N85	0.2
N86	4
N87	0.4
N88	0.2
N89	0.2
N90	0.4
N91	resistant
N92	0.6
N93	5
N94	2
N95	0.3
N96	2
N97	2
N98	0.7
N99	0.6
N100	5
N101	0.8
N102	0.2
N103	2
N104	0.7
N105	0.2
N106	0.3
N107	0.6
N108	0.3
N109	1
N110	1
N111	2
N112	5
N113	1
N114	1
N115	10
N116	0.64
N117	0.32
N118	0.4
N119	0.9
N120	1.2
N121	0.2
N122	2.5
N123	0.6
N124	4
N125	15

N126	4
N127	0.2
N128	1
N129	2
N130	0.2
N131	0.2
N132	5
N133	2
N134	resistant
N135	5
N136	resistant
N137	1.5
N138	1
N139	IC50 2
MV*	2
SD*	2.5

*Resistant and inhibitory responders are not considered

Figure 19

Approx. 200% stimulatory concentrations of the 139 plasma samples

Figure 20 illustrate the number of strong, moderate, low, resistant, and inhibitory responders of the 139 analysed individual plasmas.

RESPONDER	**N=139**
Strong responders (0.1-2.5 mg/l of gentamicin)	99
Moderate responders (2.5-6 mg/l of gentamicin)	23
Low responders (6-19.6 mg/l of gentamicin)	8
Resistant responders (>19.6 mg/l of gentamicin)	6
Inhibitory responders	3

Figure 20

Strong, moderate, low, resistant, and inhibitory responders in 139 analysed individual plasmas

Finally, figure 21 shows the distribution of strong, moderate, low, resistant, and inhibitory responders in the 139 analysed individual plasmas**.**

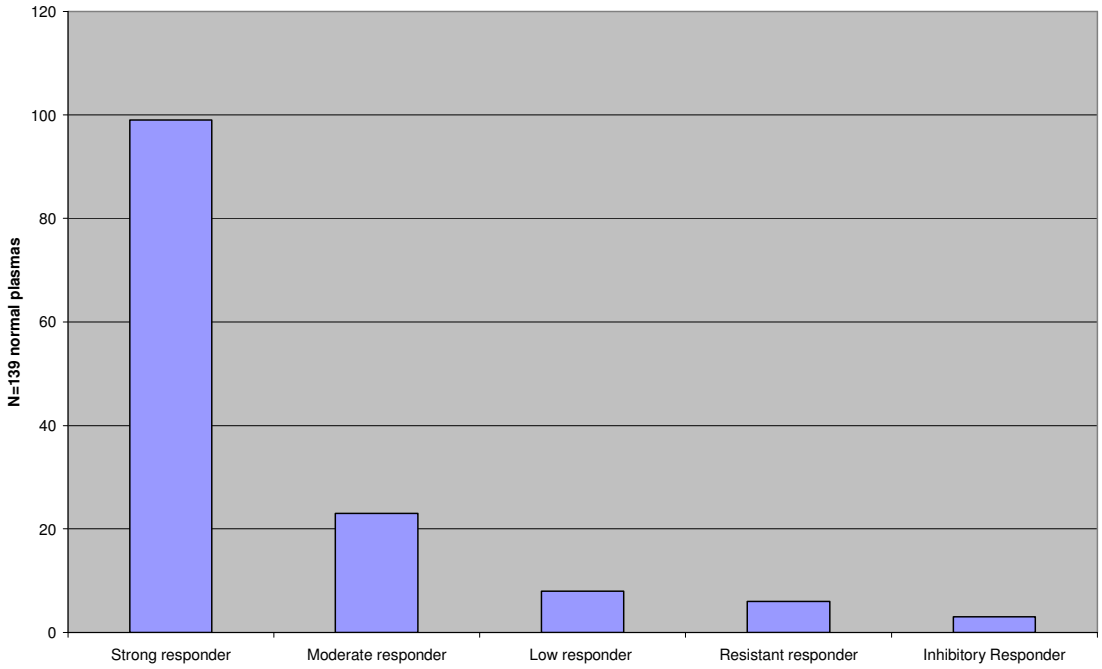


Figure 21

Distribution of strong, moderate, low, resistant, and inhibitory responders in 139 analysed individual plasmas

5. Discussion

5.1. Effects of gentamicin on intrinsic coagulation and thrombin generation

The present work demonstrates the triggering action of gentamicin on thrombin generation. The enhanced thrombin generation can be verified and quantified by the recalcified coagulation activity assay. This effect of gentamicin probably derives from its contact activation of factor 12, which leads to a thrombin generation over the intrinsic pathway of coagulation. Gentamicin folds factor 12 and/or prekallikrein into factor 12a and/or kallikrein. Factor 12a and/or kallikrein activate factor 11a, which generates factor 9a, an activator of F10a, the generator of thrombin.

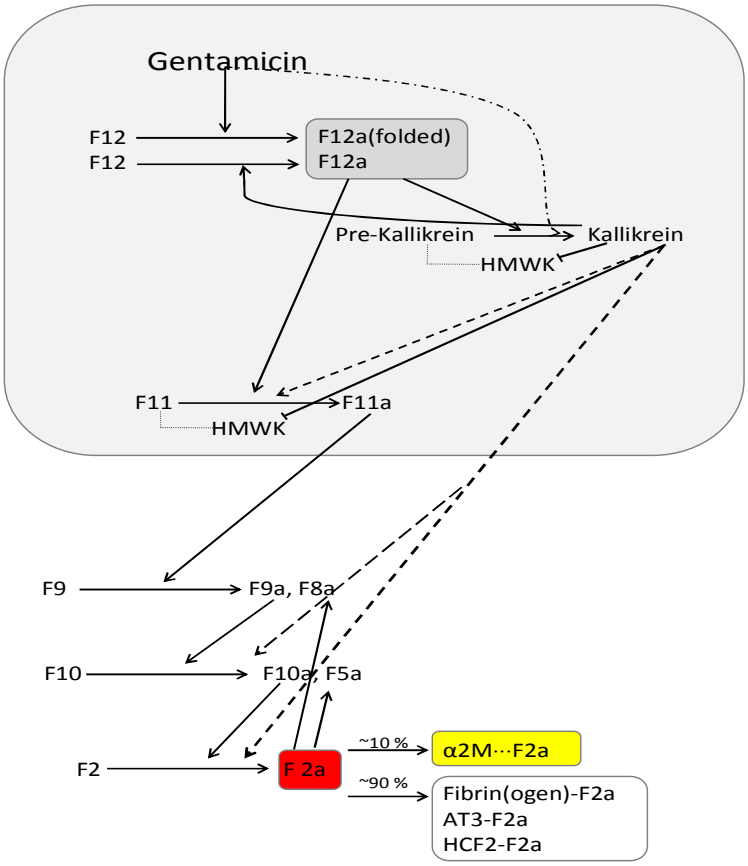


Figure 22

Triggering action of gentamicin on intrinsic coagulation

Adapted with kind permission from[91] with thanks to Christel Müller from the photodepartment of the university hospital of Marburg.

Because the therapeutic dosage of gentamicin is between 1.5 and 2 mg gentamicin/kg bodyweight[104], the described approx. 200% stimulatory concentrations are very low triggering doses. Possibly, the coagulation status of these susceptible subjects would switch from normal intravasal coagulation into pathological intravasal coagulation if treated with gentamicin in case of a bacterial infection. Thrombin activation and fibrin formation are key steps in the origin of disseminated intravascular coagulation within an infection[79].

The intrinsic pathway of coagulation is believed to be less important and not relevant for the in vivo hemostasis[24, 55]. But why do patients suffer from hemophilia A or B, the deficiency of coagulation factor 8 or 9, have such bleeding problems, if the intrinsic pathway is not relevant for thrombus formation? Factor 12 deficiency does not seem to be associated with bleeding disorders, although its deficiency or selective blockade results in protection against thrombosis, suggesting that contact activation of factor 12 is very important for thrombus formation in vivo and constitutes a new therapeutic approach to anticoagulation[22, 44, 71, 72]. Because existing anticoagulants like vitamin k antagonists are very effective as far as anticoagulation is concerned, but have seriously side effects, in 2013 van Montfoort et al. investigated, whether the selective blockade of intrinsic coagulation factors would be a superior target and would be associated with less bleeding risks than commonly used anticoagulants. They concluded that it is reasonable to practice more investigation on the blockade of the intrinsic pathway, because it is an appropriate and effective way of anticoagulation with reduced side effects, interactions and bleeding risks[95]. These aspects illustrate the importance of the intrinsic pathway of coagulation for hemostasis suggesting that factors, which can influence this system and cause hypercoagulability or bleeding disorders, are to be taken seriously, because this is very relevant for clinical practice.

In 2006 James et al. described how gentamicin decreased the partial prothrombin time and increased the plasma levels of factor 8 and factor 9 in two patients with

hemophilia A and respectively hemophilia B[40]. This supports the assumption that gentamicin can directly activate coagulation factors and by this trigger thrombin generation. Probably, gentamicin can directly activate intrinsic coagulation factors as well as activate factor 12 through contact activation.

Interestingly, ototoxicity, a serious and important side effect of gentamicin therapy, seems to be prevented by an anticoagulant therapy with aspirin[21, 80]. Sha et al. and Chen et al. believed it was because of the antioxidant effect of aspirin. Maybe the pathophysiology underlying gentamicin induced ototoxicity also includes a pathological intrinsic generation of blood micro-thrombi due to thrombin activation? Although aspirin mainly inhibits platelet aggregation and does not directly influence plasmatic hemostasis, the interactions between platelets and intrinsic coagulation factors enable an indirectly inhibition of contact activation of factor 12 and thus inhibition of thrombin generation by an aspirin induced platelet aggregation.

The pathologic thrombin generation, which is triggered by gentamicin, is a possible explanation of why gentamicin can act similarly to an endotoxin[4, 14, 19, 28, 51]. Once daily gentamicin dosing (= high plasma concentration) is thought to go along with an increased risk for endotoxin reactions[14, 28]. Fibrin, which is generated by thrombin, stimulates monocytes to produce IL-1ß via integrin receptor CD11b/CD18 and suppresses the production of IL-1 receptor antagonist, causing a systemic inflammatory reaction [3, 41, 50, 61, 66, 82, 83, 102]. Possible clinical symptoms are fever, chills, rigor, shivering, tachycardia, often with hypotension, respiratory symptoms, and muscle cramps[4].

5.2. Effects of gentamicin on platelets and possible consequences in vivo

Gentamicin does not only have effects on intrinsic coagulation, but also on thrombocytes. These effects have to be considered if the results are transferred into clinical practice. In 1997 Sakurai et al. reported how the aminoglycosides gentamicin and amikacin inhibited platelet aggregation and thus prevented EDTA-pseudothrombocytopenia for the first time[76]. In 2011 this finding was confirmed by Zhou et al. for amikacin, but not for gentamicin, although the mechanisms were still unknown[106].

In 2012 Chen et al. demonstrated a further interaction between gentamicin and platelets. In their work they showed how gentamicin inhibited the ADP- and GPIIb/IIIa-induced platelet aggregation. Furthermore they described how the partial thromboplastin time protracted with increasing gentamicin concentrations starting at 30 mg/l up to 910 mg/l, taking values of longer than 240 seconds, 10 times longer than physiologically[20]. Because the PTT measures the activity of coagulation factors 5, 8, 9, 10, 11 and 12, it seems to be in contradiction with this present work, in which gentamicin triggering action on intrinsic coagulation was demonstrated. The PTT was not measured, but the enhanced thrombin generation would rather result in an abbreviated than in a prolonged PTT. On the other hand it must be considered that Chen et al. used platelet-rich plasma mixed to platelet-poor plasma. RECA, the coagulation assay utilised in the present work, works with platelet-poor plasma. So, the effects of gentamicin on platelets and the interactions between coagulation factors and platelets were not analysed or considered. These aspects should be considered for the translation of the results into clinical application and there are therefore some reflections for that purpose illustrated by three possible situations in vivo.

The first one is: Gentamicin triggers intrinsic coagulation generating thrombin, which can be measured by RECA. Additionally, thrombocytes themselves also trigger thrombin generation, consume coagulation factors and prolong the in vitro measured PTT[88]. The measured protracted PTT is an in vitro artefact. The patient suffers from a

thrombophilic disorder like thrombus formation or pathologic intravascular coagulation. The inhibiting effect of gentamicin on platelet aggregation can be neglected, because the twofold trigger on thrombin generation is so strong that hypercoagulability does dominate.

The second reflection is: Gentamicin triggers intrinsic coagulation generating thrombin, which can be measured by RECA. Additionally, gentamicin stops the ADP- and GPIIb/IIIa-induced platelet aggregation. Because primary hemostasis is stopped, plasmatic hemostasis can not be initiated. Maybe gentamicin activates factor 12 directly and there is little thrombin generation, but the inhibition of platelet aggregation is so strong, that the anticoagulatory effect dominates. Clinically, the patient suffers from hemorrhagic disorders; he can have bleeding problems, its PTT is prolonged because plasmatic factors can not be activated due to the inhibition of platelet aggregation.

The third one is: Gentamicin massively triggers intrinsic coagulation generating thrombin, a potent activator of thrombocytes. Hemostasis is then massively activated up to overt DIC with consumption of clotting factors. The patient can suffer from massive bleeding plus intravasal fibrin clotting.

For the translation of the results into clinical application it also should be considered that the analysed blood samples derived from healthy donors with no coagulation disorders. Gentamicin is often used in septic patients, whose coagulation is already affected. They have a major risk for a disseminated intravascular coagulation and depending on the phase, they can suffer from hypercoagulability due to the activation of plasmatic coagulation and consumption of AT-3 or bleeding disorders caused by the consumption of plasmatic coagulation factors[105]. Because of the inflammation response of the organism to the bacteria and the production of acute phase proteins, also patients with a banal bacterial infection have already a changed coagulation status. Fibrinogen, an important component of human coagulation, also belongs to the acute-phase proteins and its increased production can cause hypercoagulability[29].

Keeping these reflections in mind, there are two main aspects to be considered. Firstly, gentamicin can behave differently in critical ill patients than in healthy patients as far as gentamicin induced thrombin generation is concerned. In critical ill patients the effect of gentamicin on hemostasis probably depends on many factors; e.g. on the gentamicin effects on platelets, on the pre-existing coagulation disorders caused by the bacterial infection, on the individual susceptibility to gentamicin, on the pre-existing diseases, or on a pre-existing therapy with a heparin, etc. The reactions of those patients to gentamicin can not totally be predicted because of all the different influences, which can not be reproduced in an in vitro experiment. The second aspect regards critical ill patients or patients with a bacterial infection being hypercoagulable. Is it a consequence of their pathologic blood coagulation caused by the bacterial infection and/or by a disseminated intravascular coagulation or is it a consequence of the therapy with gentamicin? However, in both cases the consequence is the same. The patient suffers from a thrombophilic disorder and needs an adequate anticoagulation.

5.3. Conclusion

Concluding, it can be affirmed with certainty that gentamicin can influence human hemostasis. This drugs effect was analysed and described by many authors. The present work demonstrates the triggering action of gentamicin on thrombin generation. Gentamicin can influence human coagulation on many ways. The in vivo effect is probably more complicated than imagined and depends on many factors like pre-existing disorders, gentamicin effects on platelets and plasmatic hemostasis, the interactions between them, and the coagulation changes induced by a bacterial infection. One possibility for the direct translation into clinical application is to analyse the effects of gentamicin on human coagulation under in vivo circumstances. It would be important to have the same conditions, although it would be not that easy. In ideal circumstances, the chosen patients have an infection caused by the same bacteria, they have the same pre-existing disorders, the same pre-treatment with heparin, the same age and gender, etc. But even in that case, the reaction to gentamicin is probably individually different. Therefore it seems more reasonable for clinical practice to control specific coagulation parameters of patients treated with gentamicin. The partial prothrombin time, the bleeding time, the international normalised ratio, and the thrombin generation should be monitored in every patient treated with gentamicin adapting the therapy to its specific condition. If there is an excessive thrombin generation, we could prove that this can be measured by the ultra-sensitive, ultra-specific recalcified coagulation activity assay and be prevented by the administration of the low molecular weight heparin enoxaparin-natrium.

The problem of thrombin generation assays is that currently there is not a standard method for the quantification of thrombin generation. There is poor standardisation and a great inter-laboratory variability. In 2004 Hemker et al. came to the resolution that methods using fluorogenic substrates were more appropriate than stirred methods[36]. In 2011 Castoldi et al. focussed on the Calibrated Automated Thrombography (CAT). Basically, fluorogenic substrate is given to whole plasma in

microtiter plates and then thrombin generation is measured with a fluorometer and converted in thrombin generation curves. The advantage of CAT is the possibility to use platelet-rich plasma and so get results closer to the in vivo situation[18]. Current studies analysing thrombin generation in different contexts also use fluorogenic methods, especially the Calibrated Automated Thrombography[12, 23, 31, 32, 56, 84]. It is difficult to affirm which method is the best. More important is to standardise a method for all laboratories. For sure, RECA is an ultra-sensitive, ultra-specific method and has to be considered for this purpose.

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7. Register of the academic teachers

My academic teachers in Marburg were Mmes/Messrs:

Adamkiewicz

Arabin

Aumüller

Bartsch

Basler

Bauer (1)

Bauer (2)

Baum

Becker (1)

Becker (2)

Becker (3)

Berger

Brehm

Burchert

Cetin

Cherkasov

Czubayko

Daut

Del Rey

Dodel

Donner-Banzhoff

Ellenrieder

Fendrich

Fuchs-Winkelmann

Geks

Görg

Gress

Grosse

Grzeschik

Häußermann

Hegele

Hertl

Heyse

Höffken

Hofmann

Holst

Hoyer

Jerrentrup

Kann

Kaufmann

Kim-Berger

Kircher

Klose

Koczulla

Koolman

Konrad

Kruse

Kunsch

Kühne

Kühnert

Leonhardt

Leube

Lill

Lohoff

Maisch

Mandic

Maschuw

Maurer

Meier

Meißner

Meyer

Michl

Moll

Mueller

Müller-Brüsselbach

Neff

Neubauer

Neumüller

Nimphius

Nimsky

Oertel

Pagenstecher

Peterlein

Pfützner

Plaut

Plöger

Preisig-Müller

Ramaswamy

Ramerth

Reese

Renz

Richter

Ries

Rosenow

Röhm

Ruchholtz

Sahmland

Schäfer (1)

Schäfer (2)

Schmidt

Schu

Schulze

Schütz

Sekundo

Seitz

Sevinc

Sommer

Steinfeldt

Steiniger

Steinkamp

Stief

Stiewe

Straßmann

Stries

Strik

Strüwer

Suske

Tackenberg

Vitelli

Vogelmeier

Vogt

Wagner

Weber

Weihe

Werner

Westermann (1)

Westermann (2)

Westhoff

Wiegand

Wilhelm

Wulf

Zettl

My academic teachers in Gießen were Mmes/Messrs:

Dettmeyer

Schneider

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