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IN VIVO BIOCOMPATIBILITY EVALUATION OF NORFLOXACIN-LOADED CHITOSAN NANOFIBERS AND BORONIC ALDEHYDE-FUNCTIONALIZED DERIVATIVES IN RAT

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IN VIVO BIOCOMPATIBILITY EVALUATION OF NORFLOXACIN-LOADED CHITOSAN NANOFIBERS AND BORONIC ALDEHYDE-FUNCTIONALIZED DERIVATIVES IN RAT (Abstract): Chitosan-based nanofibers can serve as carriers for antimicrobial agents, providing controlled drug release and biocompatibility, which are crucial for tissue integration and healing. This study **aimed** to evaluate the *in vivo* biocompatibility of norfloxacin-loaded chitosan nanofibers (NorC) and norfloxacin-loaded chitosan nanofibers functionalized with boronic aldehyde (NorCB), in comparison with standard wound treatments in rats. **Materials and methods:** NorC fibers were prepared via electrospinning of chitosan (CHIT) solution containing norfloxacin (Nor). NorCB fibers were obtained by imination of NorC fibers with boronic aldehyde. The study included four groups of Wistar rats: (i) a negative control group, which remained intact with no procedures performed, (ii) a positive control group with a simple incision covered by sulfadiazine-impregnated dressing as a clinically established reference for local antimicrobial protection, and (iii and iv) experimental groups with an incision covered by NorC, respectively NorCB nanoporous fibers. Biocompatibility was evaluated through clinical monitoring, as well as hematological, biochemical, and immunological blood analyses. All procedures were performed in compliance with institutional ethical guidelines for animal experimentation. **Results:** Both NorC and NorCB fibers did not impair liver or kidney function, did not induce local or systemic inflammatory responses, and preserved immune defense capacity. Laboratory parameters evaluated were comparable with those of the control groups. Functionalization with boronic aldehyde had no adverse impact on these outcomes. **Conclusions:** Nor-loaded CHIT nanofibers, with or without boronic agent functionalization, demonstrate good *in vivo* biocompatibility, maintaining organ function, immune competence, and absence of inflammatory effects. These findings support their potential as localized antibiotic delivery systems and advanced wound care materials. **Keywords:** CHITOSAN NANOFIBERS, NORFLOXACIN, BIOCOMPATIBILITY, RATS, 2-BORONOBENZALDEHYDE.

INTRODUCTION

Burn injuries remain a significant global health challenge, leading to high morbidity and mortality due to infection, delayed healing, and scar formation (1). Wounds resulting from burns are particularly susceptible to microbial colonization due to the loss of the protective skin barrier, creating an ideal environment for pathogenic bacteria such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* (2). Effective management of burn wounds requires dressings that not only provide a physical barrier but also deliver therapeutic agents to prevent infection and support tissue regeneration (3).

Conventional topical antibiotics have been used widely in burn care, but their effectiveness can be limited by poor penetration into tissue and the development of bacterial resistance (4). Norfloxacin (Nor), a broad-spectrum fluoroquinolone antibiotic, has demonstrated potent antimicrobial activity against Gram-negative and some Gram-positive organisms implicated in burn wound infections (5). Its mechanism of action involves inhibition of bacterial DNA gyrase and topoisomerase IV, which are essential for DNA replication (6). However, systemic use of Nor can be associated with side effects and suboptimal local concentrations, leading to interest in localized delivery strategies (7).

Advanced wound dressings based on biodegradable polymers have been developed to overcome these limitations by enabling sustained local delivery of antibiotics directly at the wound site (8). Among these polymers, CHIT, a deacetylated derivative of chitin, has attracted considerable attention due to its inherent biodegradability, biocompatibility, and intrinsic antimicrobial and hemostatic properties (9). CHIT can also promote cell proliferation and tissue regeneration, making it an attractive matrix for wound healing applications (9).

Electrospun CHIT nanofibers provide a structural architecture that mimics the extracellular matrix, enhancing cell attachment and facilitating gas exchange while also serving as a reservoir for drug loading and sustained release (10). The incorporation of Nor into CHIT nanofibers has been shown to improve localized antimicrobial efficacy and reduce infection in animal models of wound healing (11). Furthermore, CHIT-based nanofiber dressings have demonstrated improved outcomes compared to conventional gauze or hydrocolloid dressings in terms of infection control and accelerated re-epithelialization (12

Despite progress in the development of polymeric wound dressings, there remains a need for further optimization of antibiotic delivery systems that balance controlled drug release with minimal systemic exposure and maximal therapeutic effect at the injury site (13). By integrating advanced polymer science with antimicrobial therapeutics, innovative biomaterials such as Nor-loaded CHIT nanofibers hold promise for improving burn wound management and patient outcomes.

The **aim** of this research was to evaluate biodegradable CHIT-based nanoporous systems loaded with Nor for topical delivery, assessing their *in vivo* biocompatibility, biodegradability, and potential to provide sustained antimicrobial and anti-inflammatory effects.

MATERIALS AND METHODS

Substances. Norfloxacin (≥98%), Chitosan (126 kDa, 97%), poly(ethylene oxide) (1000 kDa), acetic acid (≥99%), 2-boronobenzaldehyde (97%), ethyl alcohol (98.89%), sodium hydroxide (95%) were purchased from Sigma-Aldrich Chemical Co (Steinheim, Germany). 2-boronobenzaldehyde (boronic aldehyde) was purified by column chromatography, and ethanol was dried over molecular sieves prior to use.

Preparation of composite nanofibers. The composite nanofibers were prepared through a multistep process that included the fabrication of mesoporous CHIT fibers, the incorporation of Nor, and subsequent surface functionalization via imine bond formation with boronic aldehyde.

CHIT was initially subjected to alkaline hydrolysis according to established protocols reported in the literature. Porous CHIT-based fibers were subsequently produced by electrospinning a 2.1% (w/v) chitosan/poly(ethylene oxide) blend (2:1, w/w) prepared in 80% acetic acid, using a NanoSpinner StarterKit. The poly(ethylene oxide) fraction, serving as a sacrificial component, was selectively removed by immersion in 5% sodium hydroxide solution for 2 hours. The resulting fibers were thoroughly rinsed with distilled water until neutral pH was achieved, leading to the formation of a porous fibrous network. Drug incorporation was carried out by equilibrium absorption. In brief, around 400 mg of the prepared fibers were placed in 500 mL of a saturated Nor solution and kept under sealed conditions for 24 hours to allow loading.

Following drug uptake, the fibers were recovered and allowed to dry at ambient conditions, followed by further drying under vacuum. The amount of incorporated antibiotic was determined by UV-Vis spectroscopy, using absorbance readings at 272 nm and quantification based on a previously established calibration curve.

For surface modification, the drug-loaded fibers were treated with a 0.28% ethanolic solution of boronic aldehyde (10 mL per 305 mg of fibers), maintaining a glucosamine-to-aldehyde molar ratio of 10:1. This treatment promoted the formation of dynamic imine bonds on the fiber surface.

FTIR measurements were performed to examine the chemical structure of the nanofibers and to verify the presence of newly formed interactions. Spectra were acquired in ATR mode using a VERTEX 70 FT-IR spectrophotometer (Bruker, Karlsruhe, Germany) over the 4000–600 cm^{-1} range, with 32 scans recorded at a resolution of 4 cm^{-1}. The resulting data were processed using OPUS 6.5 software.

Two distinct nanofibrous systems were thus obtained: NorC, consisting of CHIT fibers loaded with Nor, and NorCB, representing the corresponding fibers additionally modified through imination with the boronic aldehyde.

***In vivo* biocompatibility evaluation.** The study was conducted using healthy, pathogen-free, non-genetically modified Wistar rat (300–350 g), obtained from the university bio base and originally sourced from the Cantacuzino National Medico-Military Institute for Research and Development. Prior to experimentation, the animals were allowed a 24-hour acclimatization period and were housed individually under controlled environmental conditions (21±2°C, 50-70% relative humidity, 12 h light/dark cycle), with ad libitum access to standard diet and water. To minimize chronobiological variations, all experimental procedures were consistently performed between 08:00 and 12:00.

The experimental protocol comprised four groups: a negative control group (Negative C), with no intervention; a positive control group (Positive C), in which a standardized incision in the right flank region was treated with a sulfadiazinecontaining dressing as a reference for antimicrobial protection; and two experimental groups in which a similar-sized incision was performed in the same anatomical area (right flank), followed by application of NorC and NorCB nanofibers, respectively, as dressings.

In vivo biocompatibility was evaluated through the analysis of hematological, biochemical, and immunological parameters. Biochemical markers included liver function indicators (alanine aminotransferase – ALT, aspartate aminotransferase – AST, and lactate dehydrogenase – LDH) and renal function markers (urea and creatinine). Hematological assessment comprised leukocyte differentials, including polymorphonuclear neutrophils (PMN), lymphocytes (Ly), monocytes (M), eosinophils (E), and basophils (B). Additionally, immune function was assessed by measuring complement activity and the phagocytic capacity of peripheral blood PMN (NBT%), providing insight into potential systemic toxicity and immune response following treatment.

Blood samples were collected from the lateral tail vein. To minimize stress, animals were gently restrained, and the tail was briefly warmed to promote vasodilation. After local disinfection, approximately 0.3 mL of blood was drawn for analysis. Sampling was performed at two defined time points: 24 hours and 7 days after the surgical procedure and application of the test materials.

Ethical aspects. The experiments were carried out at the CEMEX Laboratory - Advanced Research and Development Center for Experimental Medicine Ostin C Mungiu, Grigore T. Popa University of Medicine and Pharmacy, Iași, following approval from the University Ethics Committee (Certificate No. 160/04.03.2022) and in strict accordance with national regulations (15) and international guidelines on the use of laboratory animals (16).

At the end of the experiments, the animals were humanely euthanized under general inhalation anesthesia with isoflurane (3% induction) using a veterinary open-circuit system (Komesaroff Mark 5, Kruuse, Odense, Denmark).

Statistical analysis. Data were analyzed using SPSS version 17 (IBM Corp., Armonk, NY, USA). Group comparisons were conducted primarily with one-way ANOVA, followed by Tukey's post hoc test when significant differences were observed. Results are reported as mean ± standard deviation (S.D.).

For comparisons between two groups, Student's t-test was applied, or the nonparametric Kruskal-Wallis test when assumptions of normality or homogeneity of variance were not met. Correlations were evaluated using Pearson's coefficient for parametric data and Spearman's rank correlation for nonparametric data. Simple and multiple linear regression analyses were performed to assess the influence of independent variables on dependent outcomes. Statistical significance was defined as $p < 0.05$.

RESULTS

Porous CHIT-based nanofibers incorporating Nor at a concentration of 4.35% were successfully obtained, alongside porous CHIT nanofibers loaded with Nor and subsequently iminated with 10% boronic aldehyde (17). The antibiotic was introduced into the fibers by immersion, ensuring an effective loading process. Further surface functionalization was achieved through the formation of dynamic imine bonds with a bioactive boronic aldehyde, reaching an imination degree of 10%, which provides a suitable balance between antimicrobial activity and biocompatibility. FTIR analysis confirmed the successful incorporation and good encapsulation of the antibiotic within the nanofibrous matrices (17).

Direct visual inspection of the incision site covered with the investigated dressings did not reveal any visible local changes. No signs of inflammation, redness, edema, or other macroscopic alterations were observed.

TABLE I.
The effects of NOR-loaded nanoporous systems on the percentage values of leukocyte formula components.

	Time points of evaluation	Leukocyte formula components (%)				
		PMN	Ly	E	M	B
Negative C	24 hours	27.8±4.21	68.2±7.67	0.3±0.01	3.5±0.2	0.2±0.01
	7 days	27.2±3.52	68.6±8.13	0.4±0.01	3.6±0.2	0.2±0.03
Positive C	24 hours	27.3±2.67	68.7±7.21	0.3±0.03	3.5±0.1	0.2±0.01
	7 days	27.8±3.55	68.2±7.45	0.4±0.01	3.4±0.1	0.2±0.01
NorC	24 hours	26.8±4.27	69.1±8.33	0.5±0.1	3.6±0.1	0.2±0.03
	7 days	27.1±3.45	68.9±8.17	0.4±0.03	3.4±0.2	0.2±0.01
NorCB	24 hours	26.7±4.17	69.3±8.43	0.3±0.1	3.5±0.1	0.2±0.03
	7 days	27.2±4.25	68.7±7.67	0.3±0.05	3.6±0.1	0.2±0.01

Values are expressed as the arithmetic mean ± S.D. of the mean for five animals per group.

The distribution of PMN and Ly in blood samples from animals treated with NorC and NorCB remained consistent with that observed in both the Negative Control and Positive Control groups across all time points assessed (tab. I).

Likewise, the relative proportions of E, M and B in these treatment groups showed no significant deviations from the values recorded in the control groups throughout the duration of the experiment (tab. I). These results indicate that administration of the Nor-loaded nanoporous systems did not disrupt leukocyte homeostasis or induce detectable alterations in the overall composition of circulating immune cells.

Administration of the Nor-loaded nanoporous systems did not produce statistically significant changes in plasma levels of ALT, AST, or LDH at either 24 hours or 7 days post-treatment (tab. II). The measured enzyme levels in these groups remained comparable to those observed in both the Negative Control and Positive Control groups, indicating that the nanoparticle formulations did not induce detectable hepatocellular injury or general cytotoxicity within the observed timeframe.

No significant changes were observed in plasma urea or creatinine levels in animals treated with NorC and NorCB compared with both the Negative Control and Positive Control groups at any of the evaluated time points (tab. III). These findings suggest that administration of the Nor-loaded nanoporous systems did not impair renal function or induce detectable nephrotoxicity during the course of the experiment.

TABLE II.

The effects of Nor-loaded nanoporous systems on the blood values of ALT, AST, and LDH

	Time points of evaluation	ALT (U/L)	AST (U/L)	LDH (U/L)
Negative C	24 hours	46.82±5.33	122.24±22.29	1289.24±115.17
	7 days	49.11±4.67	123.46±20.17	1291.45±121.21
Positive C	24 hours	48.37±4.55	121.62±21.52	1292.18±122.43
	7 days	48.55±4.41	123.18±23.67	1288.35±120.11
NorC	24 hours	47.26±4.17	122.34±22.21	1290.12±117.07
	7 days	48.38±5.82	124.54±22.45	1294.25±119.55
NorCB	24 hours	47.42±5.21	120.12±21.33	1287.42±122.82
	7 days	48.16±4.63	123.28±22.19	1293.37±118.67

Values are expressed as the arithmetic mean ± S.D. of the mean for five animals per group.

TABLE III.

Effects of Nor-loaded nanoporous systems on serum urea and creatinine levels

	Time points of evaluation	Urea (mg/dL)	Creatinine (mg/dL)
Negative C	24 hours	41.28±5.17	0.15±0.01
	7 days	42.42±4.43	0.16±0.03
Positive C	24 hours	42.64±4.55	0.16±0.01
	7 days	43.36±4.21	0.15±0.01
NorC	24 hours	43.25±4.13	0.16±0.01
	7 days	43.52±5.07	0.17±0.01
NorCB	24 hours	43.16±3.67	0.16±0.01
	7 days	44.38±4.11	0.16±0.03

Values are expressed as the arithmetic mean ± S.D. of the mean for five animals per group.

TABLE IV.

Effects of Nor-loaded nanoporous systems on serum complement activity and the NBT%

	Time points of evaluation	Complement (UCH_{50})	NBT (%)
Negative C	24 hours	51.34±4.45	13.34±1.43
	7 days	52.46±4.21	14.62±1.67
Positive C	24 hours	51.72±5.17	13.74±2.17
	7 days	53.68±4.55	14.26±1.82
NorC	24 hours	52.24±4.43	13.68±2.21
	7 days	53.18±5.15	13.46±1.29
NorCB	24 hours	53.25±4.33	14.24±1.41
	7 days	53.52±4.67	14.12±1.55

Values are expressed as the arithmetic mean ± S.D. of the mean for five animals per group.

In animals treated with NorC and NorCB, serum C levels showed no significant changes compared to the Negative Control or Positive Control groups. Measurements at both 24 hours and 7 days post-treatment were comparable across all groups, indicating that the treatments did not affect C activity (tab. IV). Similarly, the NBT% values, which reflect the oxidative burst capacity of neutrophils, remained similar to those of the control groups at both time points. Overall, these findings suggest that NorC and NorCB treatments did not induce detectable effects on these immune-related blood parameters (tab. IV).

DISCUSSION

Due to its unique biochemical features and natural ability to act against bacteria, chitosan has been widely studied for use as a wound item that brings support against infections. The successful fabrication of porous CHIT-based nanofibers incorporating Nor, as well as fibers further functionalized with boronic aldehyde, highlights the versatility and potential of this system for localized, controlled antibiotic delivery. The combination of efficient Nor loading and surface imination appears to provide an optimal balance between antimicrobial efficacy and biocompatibility, which is critical for wound healing applications.

Scaffolds help keep cells at the wound area and support them in growing, moving, and changing into different types of cells inside their three-dimensional structures. Effective wound-healing scaffolds must exhibit biocompatibility, biodegradability. Among the scaffolds, nanofiber scaffolds have become very popular because they have a large surface area compared to their size, lots of tiny spaces, and the ability to carry different types of substances.

The stable incorporation of Nor within the nanofibrous matrices suggests that the fibers can act as reservoirs for sustained drug release, providing a prolonged therapeutic effect at the wound site. This sustained release is particularly advantageous for minimizing dosing frequency and reducing systemic exposure, thereby lowering the risk of systemic toxicity. The controlled functionalization of the fiber surface with boronic aldehyde may further enhance local bioactivity by promoting favorable interactions with tissue components or modulating the release profile, without triggering adverse inflammatory or cytotoxic responses.

The absence of visible local inflammation, edema, or macroscopic tissue alterations in the treated regions strongly supports the compatibility of these materials with biological environments. This observation indicates that the fibers are well tolerated by surrounding tissues and unlikely to provoke detrimental immune reactions.

In vivo evaluation demonstrated that Nor-loaded nanoporous CHIT systems maintain excellent biocompatibility and biodegradability when applied directly over rat lesions. Importantly, no significant alterations in hematological, biochemical, or immunological parameters were observed, underscoring minimal systemic toxicity and confirming a favorable safety profile for these materials as localized drug delivery platforms (18, 19).

CHIT, a naturally biodegradable and biocompatible polymer, has been extensively employed in wound healing and drug delivery due to its intrinsic non-toxic nature, mucoadhesive properties, and capacity to support cellular adhesion, proliferation, and tissue regeneration (20). The high surface area, porosity, and interconnectivity of nanofibrous CHIT matrices not only facilitate efficient loading of therapeutic agents such as Nor but also allow for controlled and sustained release, which can be further tuned through surface functionalization.

Previous studies have delineated that Nor-loaded CHIT nanofibers enhance local antimicrobial activity while maintaining compatibility with host tissues (17, 21), consistent with the biological responses observed in our study.

These findings collectively indicate that CHIT-based nanofibers can provide an effective combination of localized antibiotic therapy, tissue regeneration support, and minimal adverse responses, positioning them as promising candidates for advanced wound management and targeted drug delivery applications (22, 23).

The absence of significant systemic inflammation or organ dysfunction in our experimental animals is consistent with previous studies in which electrospun CHIT/Nor scaffolds did not alter liver or kidney function markers in treated rats. This suggests that the embedding of Nor within a CHIT matrix does not interfere with systemic immune homeostasis or provoke adverse organ-specific effects, which is critical when considering potential clinical applications (22, 23). The maintenance of stable hematological and biochemical parameters indicates that the localized release of Nor from the nanofibers provides effective antimicrobial action at the wound site without triggering systemic inflammatory responses or organ stress.

Beyond systemic safety, CHIT nanofibers loaded with Nor have been reported to promote wound healing and tissue regeneration while simultaneously reducing infection and local inflammation (23, 24). These effects are likely mediated by a combination of the intrinsic biocompatibility and mucoadhesive properties of CHIT, which support cellular adhesion, proliferation, and extracellular matrix deposition, together with the sustained local release of Nor, which limits bacterial colonization and inflammatory signaling. The biological responses observed in our study are in strong agreement with those previously reported, reinforcing the concept that CHIT-based antibiotic delivery systems can integrate safely with mammalian tissues and actively contribute to the wound healing process.

A key aspect of this system is the controlled release of Nor from the biodegradable polymer matrices. By modulating the porosity, fiber diameter, and surface chemistry of the CHIT nanofibers, drug release can be sustained over time, maximizing local antimicrobial effects while minimizing systemic exposure and the associated risks of toxicity or dysbiosis. This controlled delivery approach ensures that therapeutic concentrations of Nor are maintained at the wound site for prolonged periods, improving infection control and reducing the need for repeated dosing.

Our observations corroborate previous reports (17, 25), demonstrating that CHIT-based nanofibers can maintain the therapeutic efficacy of Nor without eliciting systemic toxicity, thereby supporting their potential as safe and effective platforms for localized antibiotic therapy in clinical settings.

CONCLUSIONS

The present study demonstrates that NorC and NorCB, exhibit excellent *in vivo* biocompatibility after administration in rats. Covering the skin lesion with these nanofibers did not impair liver or kidney function, did not trigger local or systemic inflammatory responses, and preserved immune defense capacity. The addition of boronic aldehyde did not negatively affects these outcomes. When compared to standard wound treatments, including a sterile dressing and a sulfadiazine-impregnated dressing as a positive control, NorC and NorCB fibers showed comparable safety and tolerance.

These findings support the potential of CHIT-based nanofibers as localized antibiotic delivery systems for advanced wound care, offering controlled release of Nor while maintaining tissue compatibility and systemic safety. Furthermore, the previously established fabrication and characterization of these fibers by our research team provide a validated foundation for their further preclinical and translational evaluation.

NorC and NorCB nanofibers represent promising candidates for innovative wound management strategies, combining antimicrobial efficacy with biodegradability, biocompatibility, and immune safety.

CONFLICT OF INTEREST AND FUNDING

The authors declare that there is no conflict of interest.

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