

RHINOLOGY

Magnetic nanoparticles: a new tool for antibiotic delivery to sinonasal tissues.

Results of preliminary studies

Nanoparticelle magnetiche: un nuovo strumento per la diffusione degli antibiotici nei tessuti naso-sinusal. Risultati degli studi preliminari

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SUMMARY

Herein we examined the toxicity, penetration properties and ability of $Fe_2O_3 \cdot nH_2O$ magnetic nanoparticles extracted from silt of the Borovoye Lake (Krasnoyarsk, Russia) to bind an antibiotic. Experimental studies were carried out using magnetic nanoparticles alone and after antibiotic exposure in tissue samples from nasal mucosa, cartilage and bone (*in vitro*). Toxicity of particles was studied in laboratory animals (*in vivo*). Tissues removed at endonasal surgery (nasal mucosa, cartilage and bone of the nasal septum) were placed in solution containing nanoparticles and exposed to a magnetic field. Distribution of nanoparticles was determined by Perls' reaction. After intravenous injection, possible toxic effects of injected nanoparticles on the organs and tissues of rats were evaluated by histological examination. Binding between the nanoparticles and antibiotic (amoxicillin clavulanate) was studied using infrared spectroscopy. In 30 *in vitro* experiments, magnetisation of $Fe_2O_3 \cdot nH_2O$ nanoparticles resulted in their diffuse infiltration into the mucosa, cartilage and bone tissue of the nose and paranasal sinuses. Intravenous injection of 0.2 ml of magnetic nanoparticles into the rat's tail vein did not result in any changes in parenchymatous organs, and the nanoparticles were completely eliminated from the body within 24 hours. The interaction of nanoparticles with amoxicillin clavulanate was demonstrated by infrared spectroscopy. Positive results of experimental studies provide a basis for further clinical investigations of these magnetic nanoparticles and their use in otorhinolaryngology.

KEY WORDS: Nanomedicine • Ferrihydrite nanoparticles • Magnetic field • Antibiotics • Chronic rhinosinusitis

RIASSUNTO

Lo studio ha esaminato la tossicità, le proprietà di penetrazione e la capacità di legarsi con un antibiotico delle nanoparticelle magnetiche $Fe_2O_3 \cdot nH_2O$ estratte dal limo del lago Borovoye (Krasnoyarsk, Russia). Studi sperimentali sono stati effettuati utilizzando nanoparticelle magnetiche da sole e dopo esposizione ad antibiotico su campioni di tessuto di mucosa nasale, cartilagine ed osso (*in vitro*). La tossicità delle particelle è stata studiata su animali da laboratorio (*in vivo*). Tessuti prelevati durante interventi di chirurgia endonasale (mucosa nasale, cartilagine ed osso del setto nasale) sono stati immersi in una soluzione contenente nanoparticelle e poi esposti ad un campo magnetico. La distribuzione delle nanoparticelle è stata determinata attraverso la reazione di Perls. Dopo l'iniezione endovenosa, sono stati valutati possibili effetti tossici delle nanoparticelle su organi e tessuti nei ratti mediante esame istologico. Il legame tra nanoparticelle ed antibiotico (amoxicillina clavulanato) è stato studiato utilizzando la spettroscopia ad infrarossi. In 30 esperimenti *in vitro*, la magnetizzazione di nanoparticelle $Fe_2O_3 \cdot nH_2O$ ha portato alla loro infiltrazione diffusa nel tessuto mucoso, cartilagineo ed osseo del naso e dei seni paranasali. Con gli esperimenti, si è scoperto che l'iniezione endovenosa di 0.2 ml di nanoparticelle magnetiche nella vena della coda del ratto non determinava cambiamenti a livello degli organi parenchimatosi, e le nanoparticelle venivano completamente eliminate dall'organismo entro 24 h. L'interazione delle nanoparticelle con amoxicillina clavulanato è stata dimostrata mediante spettroscopia ad infrarossi. I risultati positivi degli studi sperimentali forniscono una base per ulteriori indagini cliniche sulle nanoparticelle magnetiche ed il loro utilizzo in otorinolaringoiatria.

PAROLE CHIAVE: Nanomedicine • Ferrihydrite nanoparticelle • Campo magnetico • Antibiotici • Rinosinusite cronica

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Introduction

The use of new drug formulations is especially important in the treatment of diseases where conventional therapy is not effective. One such example is chronic rhinosinusitis (CRS). According to the European Position Paper on Rhi-

nosinusitis and Nasal Polyp (EPOS, 2012)¹, the prevalence of CRS is increasing annually, and is now one of the most common chronic diseases in humans. For example, in Canada, the prevalence of CRS is 3.4% among men and 5.7% among women², in Belgium it is 6%³ and in Scotland reaches 9.6% of the population⁴.

The ineffectiveness of standard courses of systemic antibiotic therapy⁵⁻⁷, coupled with the doubtful efficacy of corticosteroids, especially in patients with CRS without polyps and concomitant allergy⁸⁻¹⁰, and the inability to achieve 100% success with surgery¹¹, indicate the need to develop newer and more effective treatment modalities. Magnetic nanoparticles as a targeted drug delivery tool for the tissues of nose and paranasal sinuses in CRS are a promising research direction.

Materials and methods

To determine penetration of magnetic nanoparticles in tissues, samples of nasal mucosa were taken from the ethmoidal labyrinth cells during functional endoscopic surgery, while cartilage and bone of the nasal septum were obtained during nasal septal surgery. The indications for nasal surgery were nasal obstruction secondary to CRS and significant nasal septal deformity.

For controlled delivery of nanoparticles in tissues, an external magnetic field device for low-frequency magnetic therapy “Pole 101” (EMA Factory, Yekaterinburg, Russia) ensuring continuous operation of one inductor with a magnetic field gradient of 4.6 mT/mm and magnetic induction 10.14-19.56 mT was used.

The study drug was prepared as follows: 0.125 grams of magnetic nanoparticles were diluted in 1 ml of normal saline (sterile 0.9% NaCl). The material was equally divided into three groups (20 experiments in each group).

In Group 1, mucosa, cartilage and bone tissue samples were placed in a flask with dispersed nanoparticles in normal saline for 20 minutes, washed in saline and sent for histological examination. In Groups 2 and 3, the same tissue samples were immersed in a flask with dispersed nanoparticles in normal saline and kept in a magnetic field produced by the device “Pole-101” for 20 and 40 min, respectively, before rinsing in saline and sent for histological examination.

For identification of the presence of nanoparticles in tissue samples, a specific reaction for detecting iron (reaction to Berlin blue or Perls’ reaction) was used. Therefore, tissue sections remained in 1% hydrochloric acid solution and after rinsing in distilled water they were stained again with aluminous carmine, rinsed in water and carried through ethanol, xylene and carbol-xylene. Iron deposits were coloured dark blue. After staining, tissue samples were studied under a light microscope at magnifications of $\times 100$ and $\times 200$.

To investigate the potential toxicity of magnetic nanoparticles, parenchymatous organs of laboratory animals (rats) were selected. Over a 10 day period, 0.2 ml of magnetic nanoparticles dissolved in distilled water were injected into the tail vein of rats ($n = 10$) every other day. Weight changes during drug administration as well as the animals’ eating habits were controlled daily. After 12 days, all rats were euthanised under general anaesthesia in accordance with GLP principles

of euthanasia. To determine changes in investigated organs, morphological examination assessing macroindicators was carried out. Tissue biopsies were fixed in 10% formalin solution. Slices of 5-7 μm thickness were stained with haematoxylin followed by microscopic examination of the material at magnifications of $\times 100$ and $\times 200$. All animal experiments were carried out in accordance with the requirements and provisions outlined in the Declaration of Helsinki of the World Medical Association (2000) and the local ethics committee approved the final study protocol.

Given the fact that magnetic nanoparticles of ferrihydrite are biologically neutral and may bind drugs by either covalent bonding or simple adsorption, nanoparticles were mixed with the antibiotic amoxicillin clavulanate. To confirm binding, infrared spectroscopy was used. For this purpose, powder of 1.2 g of amoxicillin clavulanate was added to 5 ml of a solution of ferrihydrite nanoparticles and magnetised in the magnetic therapy machine “Magofon-01” (Elatomsk Instrument Plant, Elatma, Russia) with a magnetic induction of 30 ± 9 mT and a frequency of acoustic range 0.02-20 kHz for

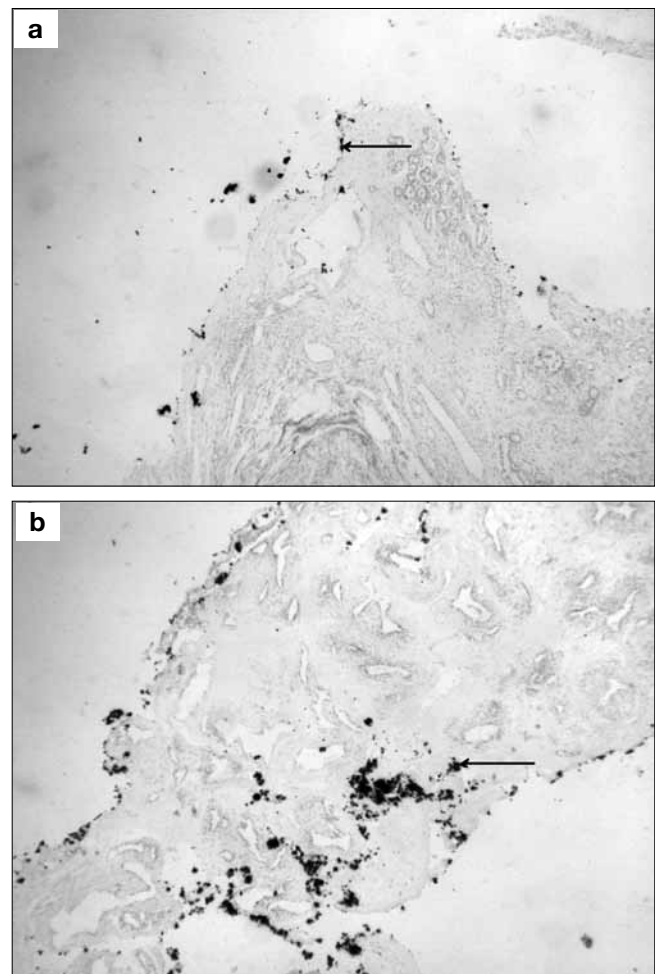


Fig. 1. Histological picture of the respiratory epithelium: a) nanoparticles (indicated by arrow) are located on the mucous membrane (Group 1); b) nanoparticles (indicated by arrows) after magnetic action, located in the nasal mucosal and submucosal layers of epithelium. Perls’ reaction. $\times 100$.

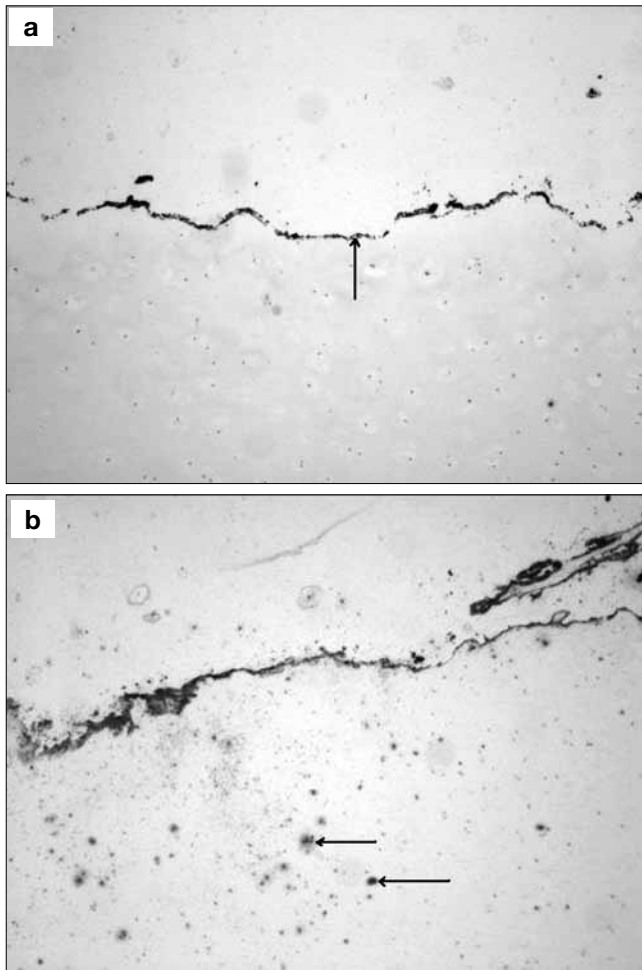


Fig. 2. Histological picture of nasal septum cartilage tissue: a) iron nanoparticles (indicated by arrow) are present only on the surface of cartilage (Group 1); b) iron-containing nanoparticles (arrows) after exposure to magnetic field are defined in the thickness of the cartilage. Perls' reaction. $\times 100$.

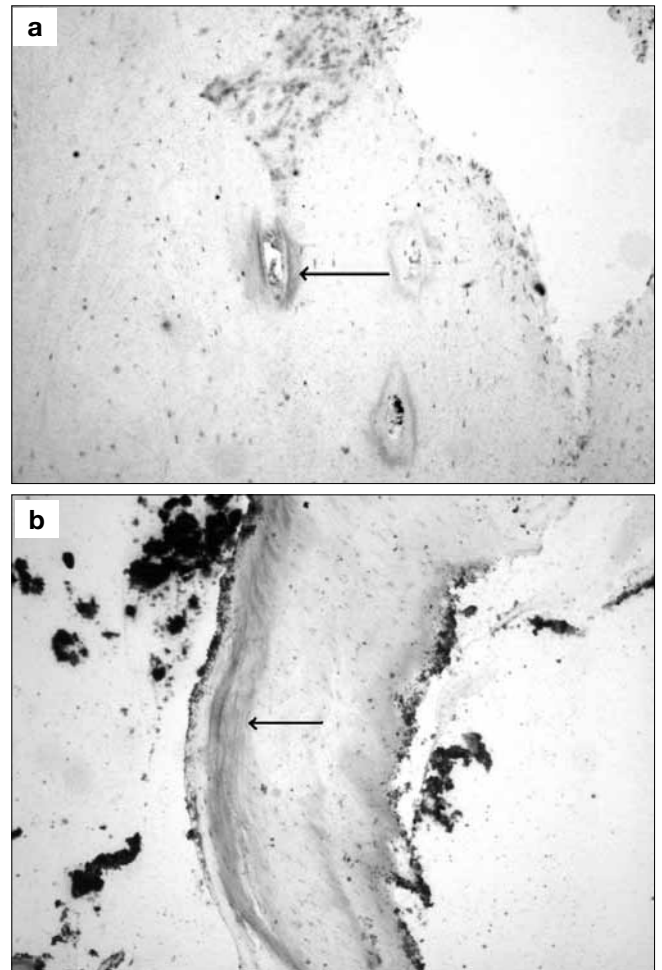


Fig. 3. Histological picture of bone tissue: a) iron nanoparticles (indicated by arrow) located on the edge of the bone channels (Group 1); b) nanoparticles (indicated by arrow) after magnetic action determined throughout the bone thickness. Perls' reaction. $\times 100$.

5 min, and placed in a liquid reservoir with BaFe_2 glasses of 0.1 mm thickness. Infrared spectrum was recorded at room temperature using Nicolet-6700 (Thermo Scientific, USA) in the range from 1100 to 2800 cm^{-1} (Group 1). A similar study was conducted with 1.2 g of amoxicillin clavulanate powder dissolved in 5 ml of ferrihydrite nanoparticles without magnetic action (Group 2) as well as with 1.2 g of amoxicillin clavulanate powder dissolved in 5 ml of water without nanoparticles (Group 3). Each group was studied with 10 identical experiments.

Results

Tissue penetration of nanoparticles (nasal mucosa and nasal septum cartilage and bone morphology)

The study of penetration of magnetic nanoparticles in tissues of the nose showed that in Group 1 nanoparticles did not penetrate deeply into the nasal mucosa, but were found only on the surface of epithelium (Fig. 1a). In addition, despite the relatively loose structure of the nasal sep-

tal cartilage, without exposure to a magnetic field, nanoparticles were located on the edge of cartilage (Fig. 2a). A similar pattern was also observed in the bone tissue of the nasal septum. Coloured nanomaterial was located mainly in the lumen and around the bony canals. Ferruginous substance did not penetrate into the bone tissue (Fig. 3a). Following the exposure of tissues to a magnetic field for 20 min (Group 2), the activity of iron-containing nanoparticles increased substantially. As a result, magnetic nanoparticles were found in the epithelium and subepithelial layer of the nasal mucosa and also sporadically dispersed (Fig. 1b). In addition, under the influence of a magnetic field gradient, nanoparticles freely penetrated into the body of the nasal septal cartilage (Fig. 2b) and were diffusely distributed. The same pattern was observed in bone tissue of the nasal septum where the nanoparticles penetrated throughout bone fragments (Fig. 3b). After doubling exposure time to the magnetic field (Group 3), there was no significant increase in the concentration of iron compounds in tissues.

In vivo analysis of nanoparticle injection in a rat model

To assess toxicity of the magnetic nanoparticles, laboratory animals (rats, $n = 10$) treated with nanoparticles were followed for 10 days and then examined histologically. Global vital parameters of the experimental animals, i.e. medium body weight, did not change significantly and eating habits remained the same over the time of observation.

Animal liver tissue. Microscopic examination revealed that the lobular-girder structure of the liver was preserved. Hepatic lobule, sinusoidal capillaries, blood vessels and bile ducts displayed normal structure in all animals. Moderate polymorphism of hepatocytes and their nuclei was observed. The cytoplasm of investigated cells was stained uniformly. Kupffer and endothelial cells had normal shape; their hyperchromic nuclei were properly located. Perls' reaction did not demonstrate any presence of iron nanoparticles in liver tissue.

Animal kidney tissue. Renal glomeruli demonstrated normal structure. Capillary loops of some glomeruli were collapsed, and nuclei were elongated. Epithelium of renal tubules was structurally normal. Clear brush rim was observed in the proximal tubules, and the lumen had normal width. Collecting glomeruli had a normal shape and contained dark and light epithelial cells. Magnetic nanoparticles in tissues of the kidney, as well as in the liver, were not detected.

Animal lung tissue. No pathological changes were observed in lung tissue. Bronchi were lined with ciliated epithelium. Peribronchial accumulations of lymphoid cells with the formation of solitary lymphoid follicles were noted. The pulmonary vascular bed was anaemic. Alveoli were well inflated; small foci of atelectases were found. In Perls' reaction, nanoparticles in the lung tissue were not present.

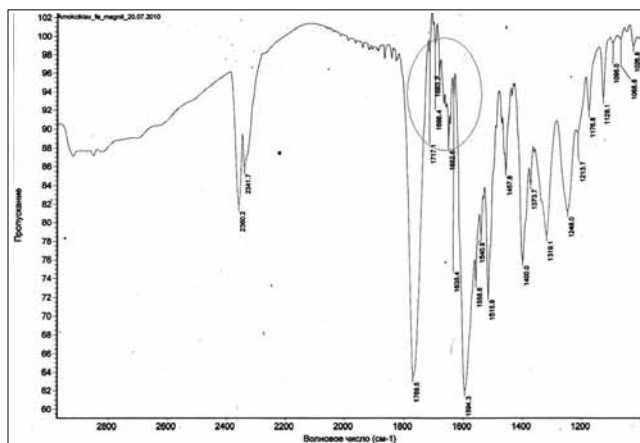


Fig. 4. Infrared spectrometry of amoxicillin clavulanate: a) dissolved in distilled water; b) associated with nanoparticles after magnetic action (changes in the spectrum are circled). Axis of abscissas, wave number (cm^{-1}); ordinate, %.

Conjugation of nanoparticle to antibiotic

When comparing the results of infrared spectroscopy of the complex nanoparticle/amoxicillin clavulanate without magnetic action and conventional aqueous amoxicillin clavulanate, no significant differences were found. However, after introduction of the complex into magnetic field, there was sharp decrease in absorption at $1650\text{--}1670\text{ cm}^{-1}$ with the appearance of additional bands at 1558.6 and 1540.9 cm^{-1} (Fig. 4a-b).

Results of all experimental studies conducted are summarised in Table I.

Discussion

One of the most rapidly developing and promising fields of scientific research to date is nanotechnology^{12,13}. This technology explores the properties and practical applications of nanoparticles which are extremely small in size

Table I. Experimental studies on magnetic nanoparticles $\text{Fe}_2\text{O}_3 \cdot n\text{H}_2\text{O}$.

Purpose	Methods	Result	Conclusion
I Investigation of penetration ability of $\text{Fe}_2\text{O}_3 \cdot n\text{H}_2\text{O}$ nanoparticles into human tissues	Tissue samples (mucosa, cartilage and bone tissue of the human nose) were kept in a flask with dispersed nanoparticles in normal saline under the action of a magnetic field or without it	Magnetisation of nanoparticles for 20 min results in their diffuse infiltration into the mucous membrane, cartilage and bone tissue	Ability to control distribution of the nanoparticles in the humans nasal mucosa, bone and cartilage by an external magnetic field
II Assessment of the toxicity of magnetic nanoparticles in laboratory animals	Injection of magnetic nanoparticles dissolved in distilled water into the tail vein of rats ($N = 10$) over a 10-day period followed by morphological examination of tissue biopsies	No pathological changes in the parenchymatous tissues (liver, kidney, and lung). Complete clearance of nanoparticles from the body within days	Magnetic nanoparticles are nontoxic
III Investigation of the ability of magnetic nanoparticles to interact and bind with an antibiotic	Infrared spectroscopy: a magnetised solution of amoxicillin clavulanate mixed with ferrihydrite nanoparticles (Group 1), an amoxicillin clavulanate + nanoparticles solution without previous magnetic action (Group 2) and an amoxicillin clavulanate solution alone (Group 3)	The change of the absorption spectrum of magnetised complex ferrihydrite/amoxicillin clavulanate at the range of $1650\text{--}1670\text{ cm}^{-1}$	Formation of a complex nanoparticles + antibiotic by hydrogen bonds or weak induction forces

(a few nm); a variety of physical and chemical substances have been examined: silicon, gold, zinc, iron, aluminosilicates, ferromagnetic and diamagnetic materials^{14 15}.

The most promising direction for nanotechnology in medicine is the use of magnetic nanoparticles as carriers of drugs (vehicles). This is due to the ability of drugs immobilised on the surface of magnetic nanoparticles to selectively concentrate and move under the influence of an external magnetic field in a specific body area¹⁶⁻¹⁸.

In 2006, magnetic nanoparticles of bacterial origin were developed in the Institute of Biophysics (Siberian Branch of the Russian Academy of Science, Krasnoyarsk, Russia). They were synthesised by cultivating gram-negative bacilli from the Enterobacteriaceae family (genus *Klebsiella*, type *Klebsiella oxytoca*) isolated from silt (sapropel) of the lake Borovoye, Krasnoyarsk District, Russia. Based on comparison of the results of magnetic and direct structural methods, the resulting magnetic nanoparticles are ferrihydrite $\text{Fe}_2\text{O}_3 \cdot n\text{H}_2\text{O}$ as seen in Figure 5. It was also demonstrated that the ferrihydrite nanoparticles 2-5 nm in size produced by *Klebsiella oxytoca* have an effective magnetic charge enabling magnetic control of these nanoparticles¹³.

Ferrihydrite nanoparticles produced by *Klebsiella oxytoca* possess unique magnetic properties in the biomineralisation of natural iron salt solutions. Antiferromagnetic order inherent in the massive ferrihydrite and spontaneous magnetic moment due to decompensation of the spins in the sublattices coexists in these nanoparticles. Enhanced by the effect of superantiferromagnetism, the magnetic susceptibility enabled magnetic control of these materials. Intravenous injection of magnetic nanoparticles in laboratory animals (rats) for 10 days caused no pathological changes in the parenchymatous tissues (liver, kidney, and lung). Absence of changes in the kidney histological architecture of these animals demonstrates that these magnetic nanoparticles were nontoxic. In addition, the study showed complete clearance of nanoparticles from the animals' body within days, characterised by negative Perls' reaction in all animal tissues studied.

The study of magnetic nanoparticles penetration into nasal tissues *in vitro* showed that the effect of an external magnetic field for 20 min resulted in a diffuse distribution of nanoparticles in mucosa, cartilage and bone tissue of the human nose. Therefore, the possibility of targeted delivery of nanoparticles into the lamina propria of the nasal respiratory epithelium is especially important in the treatment of CRS when chronic inflammation affects primarily the mucous membrane of the nose and paranasal sinuses. Penetration of iron nanoparticles into tissues confirmed their magnetic reactivity and strongly suggests that these iron nanoparticles can be controlled by an external magnetic field.

Although there is an absence of evidence-based literature that topical application of antibiotics is effective in the treatment of CRS⁷, bacteria are believed to universally present in CRS. Certain bacteria likely influence mucosal

inflammation in the paranasal sinuses contributing to CRS persistence and severity^{19 20}. Under certain circumstances, at least in case of acute exacerbation, antibiotics can ameliorate the severity of CRS. Dependence of treatment outcome on contamination of the sinuses with bacteria like *S. aureus*, and the need for pathogen eradication has been also stressed²¹. However, the current options in topical antimicrobial therapy and methods for delivery of antimicrobial agents to the paranasal sinuses are few at best²²⁻²⁴. Therefore, our first step toward future clinical implications of the new method of drug delivery was investigation of possible conjugation of a nanoparticle to antibiotic.

Preliminary results of our study have shown that the antibiotic molecule readily links with nanoparticles. Infrared spectrometry revealed the change of the absorption spectrum of magnetised complex ferrihydrite/amoxicillin clavulanate, which reliably indicates the formation of bonding between the initial reagents with participation of either hydrogen bonds or weak induction forces.

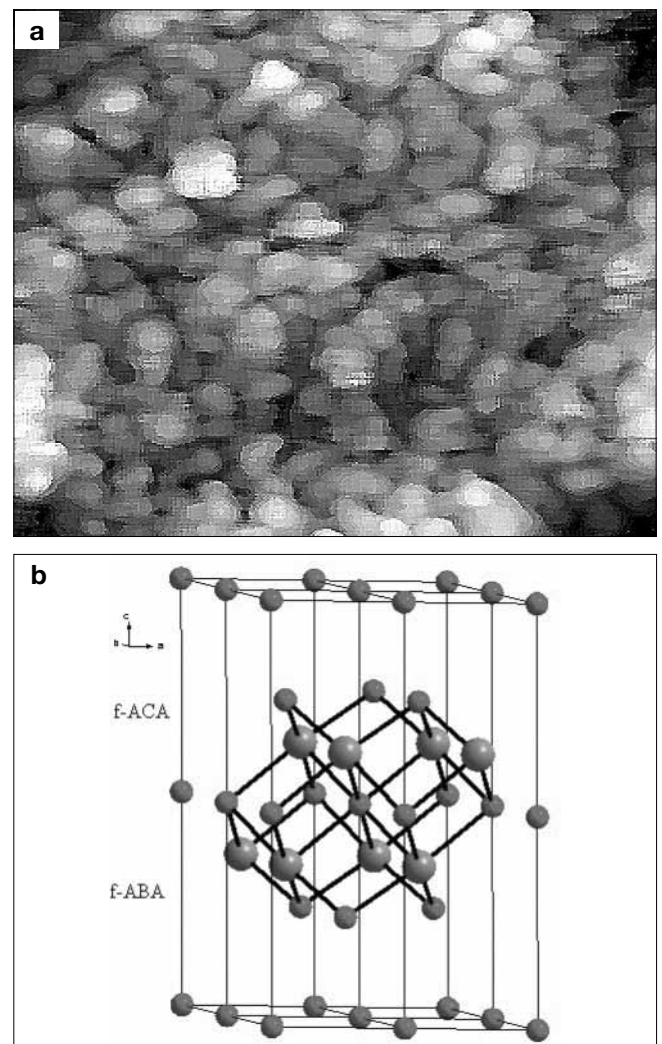


Fig. 5. Magnetic nanoparticles Fe_2O_3 : a) scanning tunnelling microscopy, $\times 20$ nm; b) schematic drawing of Fe-O-Fe links in a non-defect phase: smaller grey balls, O and OH ligands; larger grey balls, Fe^{3+} .

Conclusions

We believe that the initial results of these experimental studies provide a rational basis for development of a new method of targeted drug delivery to tissues of the nose and paranasal sinuses. Further experimental and clinical investigations of magnetic Fe₂O₃ nanoparticles linked with an antibiotic, glucocorticosteroids, or some other agents are needed, as they can considerably improve our therapeutic options in treatment of chronic inflammation of the sino-nasal mucosa.

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