Endoscopic ultrasound guided injection of iron oxide magnetic nanoparticles for liver and pancreas: a feasibility study in pigs.

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Abstract

Aims: Pancreatic cancer and hepatocellular carcinoma are two of the most aggressive types of cancer with limited therapeutic options in stages of advanced disease. Our objective is to assess the safety and feasibility of injecting iron oxide nanoparticles (IONs) via endoscopic ultrasound (EUS)-guidance, both systemically and locally in the liver and pancreas in order to study new potential therapies for liver and pancreatic tumors. **Material and methods:** Six domestic pigs were used for our study design, and divided into three groups: two were injected in the portal vein, and other four were subjected to local exposure of IONs in the liver and pancreas, two each. The pigs were on a 7 days follow-up and necropsy was performed with their organs harvested. A 3T MRI scan was also performed. **Results:** All animals underwent an endoscopic ultrasound fine needle injection (EUS-FNI) procedure without any complications. EUS-FNI procedure had an average time of 5 minutes and 21 seconds and consisted of 2 ml of ION injection. No perforations and no risk of potential bleeding were recorded. Macroscopic changes were observed only after pancreatic EUS-FNI. A significant amount of IONs was observed in the liver after local injection and after vascular EUS-FNI. The imaging results were confirmed by pathological examination with most of the IONs accumulated in Ito-like cells, Kupfer cells, and sinusoids. **Conclusions:** IONs have been widely studied for both diagnostic and therapeutic purposes. Their injection through EUS-guidance may develop new diagnosis strategies as well as curative or palliative therapies in pancreatic and liver tumors.

Keywords: endoscopic ultrasound–fine needle injection, iron oxide nanoparticles, liver, pancreas

Introduction

Pancreatic cancer (PAC) and hepatocellular carcinoma (HCC) are two of the most aggressive malignancies with a grim prognosis, ranking on the 3rd and 4th place in terms of mortality worldwide [1]. Because both diseases are frequently diagnosed in advanced stages (either local

or metastatic), curative options may be no longer available. This has brought forward the need of new potential therapies, which may prolong the patients' life expectancy and improve their conditions [2,3].

Over the years, nanotechnology biomedical applications have opened a window in the research of new diagnostic and therapeutic settings for HCC and PAC [4,5]. Among the different nanoparticles available, iron oxide nanoparticles (IONs) have received extensive attention due to their availability and properties. From diagnostic procedures in enhanced magnetic resonance imaging (MRI) to local therapies such as magnetic hyperthermia or even as vectors for shipping cytotoxic drugs, IONs have proven to have a good safety profile [6]. A key feature for their biocompatibility and biodistribution is related to their general characteristics of physiochemical design and coating properties [7].

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Regardless of their purpose and method of distribution, either oral, local, or intravenously, the main objective is to deliver a number of IONs as large as possible in the targeted tissue. Several organs, along with the pancreas and liver can become deposit spaces as the iron may be encapsulated within their cells.

Endoscopic ultrasound (EUS) has evolved since its beginning to a routinely used procedure, which further blends interventional radiology techniques with minimal invasive surgery options. Real time imaging of the pancreas and liver provides important details about the tumor's characteristics and may also serve as a precise setting for guiding therapeutic techniques [8]. After introducing EUS-guided fine needle aspiration, more procedures have surfaced expanding the therapeutic armamentarium of endoscopic therapies [9,10]. EUS-guided interventions pose a great potential with a prospect of successful treatment results as compared to current available techniques.

Development and improvements in cancer research have indicated EUS-guided fine needle injection (EUS-FNI) as a novel technique for local delivery of specific drugs [11]. The aims of the present study are to assess the safety and feasibility of injecting IONs via EUS-guidance, both systemically and locally in the liver and pancreas in order to study new therapies for liver and pancreatic tumors.

Materials and methods

The procedures were performed according to the European Legislations on animal rights, after obtaining a written approval from the Ethics Committee of the University of Medicine and Pharmacy of Craiova (UMFCV).

IONs were synthesized at the Institute of Macromolecular Chemistry "Petru Poni", Iasi, through the co-precipitation method with citric acid solution. At first, 0.60 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ was added to 2 ml deionized water and another solution was prepared by adding 0.21 g of $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ to 0.5 ml of 2 M solution of HCl. These substances were vigorously stirred after being added to 10 mL of DI water with citric acid. The resulting solution was titrated with 2 ml of 5 M of sodium hydroxide and stirred for 30 minutes until a black precipitate was formed, resulting in the Fe_3O_4 nanoparticle suspension. The ferrofluid was heated to 80°C , left for 2 hours and then centrifuged for 5 minutes at 900xg. As a final step the supernatant was added into water, a process which was repeated several times. Before being injected, the magnetic nanoparticle (MNP) solution was sonicated for a proper dispersion for several minutes.

Six domestic pigs were used for our study design and kept in special conditions. The experimental models were

subjected to fasting and liquids for 24 hours, respectively 6 hours before intervention. Premedication was administered intramuscularly and consisted of Ketamine 20 mg/kgc (MSD Animal Health, Germany), Xylazine 2mg/kgc (Bioveta A.S., Czech Republic) and Atropine 0.015 mg/kgc (Biofarm, Romania). Peripheral access was assured with an 18 G catheter (WellcathPlusTM, Wellmed, Noida, India) positioned on the marginal vein of the ear. The pigs were intubated, maintained under general anesthesia with Propofol 0,5 mg/kgc (Fresenius Kabi Austria GMBH – Austria) continuously, Fentanyl 3 $\mu\text{g}/\text{kgc}$ (Actavis Nordis A/S – Denmark) and Pavulone 0,1 mg/kgc (Pancuronium Bromide, Schering-Plough – USA) while all vital signs were monitored.

All procedures were performed with standard equipment intended for animal use only. The pigs were divided into three groups: two were injected in the portal vein, and other four were subjected to local exposure of IONs in the liver and pancreas, two each. EUS-guided injection of IONs was carried out with a linear array EUS scope (GFUCT140-AL5, Olympus, America), with a large interventional channel, coupled with a corresponding Evis Exera System (Olympus, America) and an AlokaPro-Sound 5500 Ultrasound System (Hitachi-Aloka, Tokio, Japan). EUS-FNI through a 19-gauge needle was preferred for local and portal vein injection. The EUS-scope was passed through an overtube placed into the esophagus and advanced to the stomach until a good position of the targeted organs (liver or pancreas) was obtained (fig 1).

A 19-gauge EUS needle (Boston Scientific, USA) was inserted through the biopsy channel, and 2 ml of MNP solution was directly injected either in the portal vein or directly in the liver or pancreas using real time EUS guidance. For the liver, MNP were injected in the left lobe, while for the pancreas the MNP were directed to the head region. Portal vein EUS-FNI consisted of puncturing the vascular wall under real-time

Fig 1. Study design for EUS-FNI in the pancreas on a pig model

Fig 2. a) EUS-FNI of IONs in the left liver lobe, showing a wide spreading of the ferrofluid solution around the injection area as a hyperechoic mass; b) EUS-FNI in the portal vein pointing out the IONs being dispersed by the bloodstream; c) local EUS-FNI in the head of the pancreas showing hyperechoic images as small quantities of IONs.

EUS guidance and releasing the MNP solution into the bloodstream (fig 2).

The pigs were followed for the next 7 days, with close monitoring regarding any change in their behavior, food intake and body temperature. Animals were euthanized with a pentobarbital overdose and necropsy was performed with their liver and pancreas and other organs being harvested. Kidneys and spleen were also collected to compare the quantity of additional deposits of MNPs on vascular EUS-FNI to local organ injection. Gross examination was performed and organs were stored in buffered neutral formalin and sent for a 3T MRI (Philips Ingenia 3T, Netherlands) scanning with a special research coil.

After routine processing for paraffin embedding, 4µm-thick sections were cut from the tissue blocks and were further utilized for hematoxylin-eosin and Prussian blue staining in order to assess the histopathology and ferrous iron deposition. For Prussian blue staining, the slides were incubated in a 2% potassium ferricyanide acidic solution for 30 minutes at 37°C, after which

Fig 3. Image processing for spectral unmixing separates signals by unmixing the visible light into 10nm bands spectra. Red and blue spectra cubes have been defined (a), and these signatures have been used through the analysis. An exemplary RGB image (b) is shown in pure red and blue signals (c), as well as with individual red (d) and blue channels (e).

they were counterstained with Nuclear Red, dehydrated, cleared and mounted with a xyle-based mounting medium. In order to best separate the Prussian blue and still be able to investigate the morphology of the tissue, the transmission light spectra of the Prussian blue and Nuclear Red were separated by spectral unmixing using a Nuance FX multispectral camera capable of resolving 420-720nm spectral range with a resolution of 10nm, and the Nuance 3.0.2 software (PerkinElmer, Hopkinton MA, USA) (fig 3). Blue signal areas were next calculated on 40× images centered on either portal spaces or centrilobular veins, averaged (10 image captures for each anatomical region) and means plotted and compared (Microsoft Excel, Microsoft Office 2010).

Results

All 6 pigs, with a weight range between 25-35 kg underwent a EUS-FNI procedure without any significant changes in their behavior or any sign of additional complications. No difficulties were encountered in identifying the vascular structures and the targeted organs under EUS-guidance. EUS-FNI procedure had an average time of 5 minutes and 21 seconds. During local EUS-FNI, a hyperechoic mass was created at the injection point, having a median diameter of 1.5 cm in the liver and 1.2 cm in the pancreas. After catheterization of the portal vein, the MNP solution was dispersed into the bloodstream with no immediate sign of thrombosis. No perforations of the gastric wall or other organs and no risk of potential bleeding were recorded during the procedures.

Necropsy results showed no signs of local or distant complications. Macroscopic changes were observed only after local injection in the pancreas with a black spot highlighting the concentration point where the MNP were injected (fig 4).

Fig 4. Necropsy reveals a large deposit of IONs as a black spot at the injection point in the head of the pancreas (arrow)

Fig 5. 3T MRI images recorded after organ's harvesting pointing out deposits of IONs within the liver a) and the pancreas b).

The organs were evaluated by 3 Tesla (3T) MRI scanning, which hallmarked MNP concentrations in the liver and pancreas. A significant amount of IONs was observed in the liver after local injection and after vascular EUS-FNI. Portal vein injection showed no sign of thrombosis, even though there were deposits of MNPs even in the periphery area (fig 5a). Regarding the pancreas, MRI images were only relevant after local EUS-FNI procedures (fig 5b).

Pathological assessment showed various deposits within the selected organs. Local injection in the liver showed a large volume of MNPs in the targeted area and several deposits in small quantities further away diffusing into the parenchyma. In contrast, EUS-FNI in the portal vein revealed a large amount of IONs scattered in the hepatic lobules, as well as between the lobules and in the hepatic periportal space.

Initial hematoxylin and eosin staining revealed clear-cut iron pigment deposition in the portal veins and some of the peripheral sinusoids (fig 6a), with no visible hemosiderin accumulation in and around the central veins (fig 6 b,c). More than 80% of all the visualized portal spaces presented this accumulation. However, when we utilized the specific Perl's Prussian Blue histological staining, it became clear that the extent of deposition was much larger, with diffuse depositions in the lobule around the central vein (fig 6 d,e,f), the density of the deposits increasing gradually with shorter distances to the portal spaces (fig 6 g,h). The pigment was so dense in the portal vein, that the spectral unmixing visualized the reddish appearance under the blue dense shade of Perl's staining. None of the intralobular deposits showed the same bi-phasic spectral signature due most probably to

Fig 6. Pathological assessment of the iron deposition in the liver. Only dense hemosiderin pigment is identified on hematoxylin and eosin, mainly in and around the portal spaces (a), while almost no pigment can be identified around the centrilobular spaces (b-c). Spectral unmixing on Perl's stained slides reveals more diffuse depositions around the central lobular areas (d,e,f) compared to peripheral areas (g,h,i). Most of the diffuse depositions appeared to be accumulating in Ito-like cells (j, j1), Kupfer cells (j, j2) and in the sinusoids (j, j3).

the dilution of the particles downstream the blood flow. High resolution images revealed that most of the diffuse deposits in the lobule were present in Ito-like cells (fig 6 j₁), Kupffer cells (fig 6 j₂) or in the sinusoid themselves (fig 6 j₃). Only on rare occasions it seems that the deposits occurred in the hepatocytes.

When we evaluated the blue areas in Perl's staining, this showed that despite the fact that the densest deposits were situated around the portal spaces, these were not the largest. This analysis showed that blue areas around centrilobular spaces were significantly larger ($27019.85 \pm 3290.38 \mu\text{m}^2$ and $15589.53 \pm 6526.35 \mu\text{m}^2$) than those around the periportal areas ($3290.38 \pm 195.16 \mu\text{m}^2$ and $6526.35 \pm 290.62 \mu\text{m}^2$), and this staining decreased from perihilar regions compared to peripheric lobar areas. Periportal regions had no such variation between the two anatomical sampling sites, and all instances showed significantly higher values compared to the control corresponding liver areas (fig 7).

Local pancreas EUS-FNI showed a large area of MNP deposits with a localized inflammatory tissue reaction at about 2 cm away from the injected area. A mild inflammation of the pancreatic parenchyma with fat necrosis and atrophy characterized the surrounding tissues.

Fig 7. Semiquantitative analysis of Perl's staining shows decreasing staining areas from the hilar to peripheral lobar regions. At the histological level, the areas were much larger around the centrolobular spaces compared to the periportal regions, and the difference was significant only for hilar anatomical regions. All injection sites demonstrated a larger signal area compared to the control tissue. * represents a significance on Student's t testing of $p < 0.05$. Bars represent standard error of the means.

Discussions

Treatment options for patients with liver and pancreatic tumors are known to be relatively limited if the disease is diagnosed in an advanced stage. Lately, magnetic nanoparticles have served as diagnostic tools in contrast imaging MRI in humans [12] and as therapeutic methods such as hyperthermia, drug carriers or gene therapies in experimental models [13]. Tissue reactions to MNP's physico-chemical properties are known to be influenced by their general properties and coatings, biocompatibility and toxicity and nonetheless methods of distribution [14]. Depending on their purpose they have been injected locally within a region of interest, orally after gavage following the absorption route or intravenously trying to direct them to the tumor. However, these techniques are rather difficult to control as the biological response of the targeted tissue requires a large amount of MNPs to fulfill the therapeutic effect [15]. Generally, vascular injection and distribution of MNPs require their passage through three phases: blood stream clearance, extravasation and interstitial space depositing from where they may attack the cancer cells. So far, there are few studies that exploit the potential of nearby vascular structures injection mainly because of their difficult accessibility. EUS may be able to fill up this gap, because of its great imaging potential and various therapeutic options.

Our study focused on the distribution of MNPs through several methods of injection trying to emphasize their liver and pancreas enhancement. EUS-FNI may be taken into consideration as a palliative option either by local injection or by gaining vascular access. Its potential in diagnostic and therapeutic settings based on real-time

imaging may overcome the flaws of interventional radiology. There is no doubt that EUS-guidance provides a more attractive option for vascular therapies, as both major and smaller vessels near the gastrointestinal tract can be traced and easily accessed.

EUS-FNI has the ability to target the liver and pancreas using a transgastric and transduodenal approach. So far this setting has been tested with intratumoral ethanol injection [16,17] and chemotherapeutic drugs [18]. EUS-FNI ethanol injection in the pancreas in a porcine model has been proven to be technically possible and rather safe with mild side effects [16]. Our results after local MNP injection in the pancreas showed no evidence of an important pancreatic reaction confirming that EUS-guided injection is technically successful because of the nearby position of the pancreas near the gastric wall. Similar results were noted by Kai et al [19] who injected an OncoGel solution in a porcine pancreas tail and observed the deposits 14 days after exposure proving that the technique is feasible and could be a potential therapy for advanced pancreatic cancer.

Liver delivery of MNP was assessed while using different ways of administration. EUS-FNI local delivery showed a large amount of MNP concentrated in the targeted area with only small concentrations in the surrounding liver tissue. Even though there are concise protocols for patients with HCC tumors, the use of EUS-FNI has been noted so far in animal settings, as well as some case reports [20,21]. On the other hand vascular access in the portal vein and delivery of MNP showed a wider diffusion of MNP in the entire liver, up to the distal branches. This setting seems to be a more appropriate option if a larger number of tumors are targeted.

Local EUS-FNI and portal vein EUS-FNI might be used for different tumor types. While local injection may be addressed to a solitary liver tumor (such as HCC or solitary metastases focusing on a larger concentration of MNP in the region of interest), the potential of portal vein injection could be directed in hepatic metastases covering most of the liver tissue. Also, specific coated MNP with different chemotherapeutic drugs injected through the portal vein could lower the systemic toxic effects caused by peripheral injection. This setting was studied by Faigel et al [22] who compared the level of several chemotherapeutic drugs after injecting them in the portal vein and systemically in several experimental pig models. After comparing the results he observed that irinotecan, doxorubicin loaded microbeads and paclitaxel nanoparticles had higher concentrations in the liver tissue after a portal vein injection as compared to a systemic injection, while toxic levels were almost halved especially in the cardiac tissue. MRI images were consistent

with the pathological examination when comparing local EUS-FNI to portal vein access.

This study has several limitations consisting of the small number of animals and the fact that the MNP distribution was assessed only with MRI scanning and pathological examination. While trying to validate the EUS technique we did not measure the iron concentration within the tissues and also did not focus on urinary samples to see the MNP elimination. Nevertheless, the MNP concentration can be easily estimated through imaging, leading to a better quantification inside the desired region of interest that will be targeted through magnetic hyperthermia.

Conclusions

Our study focused on showing the feasibility of EUS-FNI of MNPs in the liver and pancreas by local or vascular access and their organ distribution. EUS-guidance offers a unique access to these organs and nonetheless to nearby vascular structures, which facilitates desirable therapeutic techniques, which have been the target of interventional radiology over the years. MNPs have been widely studied for HCC and PAC in different therapeutic scenarios such as hyperthermia or coated with different chemotherapeutic drugs. Their injection through EUS-guidance may develop new strategies of diagnosis or curative or palliative therapies in pancreatic and liver tumors.

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Conflict of interest: none

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