Highly selective behaviour of gastric adenoma after administration of EMR composition and its HCT116-based model

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Article

Keywords: Adenoma, Adenomatous Polyps, Endoscopic Mucosal Resection, HCT 116, alpha-amylases

DOI: https://doi.org/10.21203/rs.3.rs-631801/v1

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Title

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Conflict of interest

We declare that none of the authors have competing financial or non-financial interests as defined by Nature Portfolio.
Data availability

The authors declare that the data supporting the findings of this study are available within the paper. The additional data that support the findings of this study are available from the corresponding author upon request.

Financial support

The authors would like to acknowledge following grants and project contributions:

FP7-REGPOT-2012-2013-1 call - Proposal 316310 - CELIM "Fostering excellence in multiscale imaging", Faculty of Science, Pavol Jozef Šafárik University in Košice

Open scientific community for modern interdisciplinary research in medicine (OPENMED), ITMS2014+: 313011V455 supported by the Operational Programme Integrated Infrastructure, funded by the ERDF Charles University grant (SVV 260 551)

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Abstract

When applying improved composition of solution used during endoscopic mucosal resection (EMR), we have observed unexpectedly large and quantitatively significant difference in response of adenoma vs. healthy tissue of surrounding GIT tract, namely the selective reaction enhancing the volume and differentiated colour. The *in vitro* experiments on model neoplasia cell line HCT116 suggest, that the robust differences in response of starving cells can be traced down principally to the tetrastarch digestion of neoplastic tissue and enhanced metabolic rate of neoplastic cells. The neoplastic tissue grows into several intestine layers so that submucosal injection of iso-oncotic tetrastarch compound leads to degradation of starch and production of oncotic molecules in submucosa transported by facilitated transport into neoplastic tissue. The colour distinction of the reporting dye is due to concentration differences of three separated compartments, further enhancing the utility of the contrasting mixture. The diffusion dynamics shall be tuneable by optimizing starch composition improving desirable pharmacokinetics.

Keywords

Adenoma, Adenomatous Polyps, Endoscopic Mucosal Resection, HCT 116, alpha-amylases.
**Introduction**

Neoplasms of the gastrointestinal tract are tumour-transformed tissues which, without treatment, can gradually progress into malignant tumours. The therapy of such neoplasms is usually radical, by surgical procedure-incision of detected neoplasms. The position, shape and size of the neoplasm is typically corroborated using the endoscopic method. The surgeon uses endoscopic optical probe to localize the position of such polyps along the gastrointestinal tract.

The neoplasia which is not embedded into the deeper layers of the tissue can be removed during the same endoscopic examination session by using polypectomy. By resection through the middle or deeper part of the submucosal layer, endoscopic mucosal resection (EMR) allows complete and curative resection of the diseased mucosa. For indicated early stages of progression, the EMR can be accomplished with minimal cost, morbidity, and mortality, and with the potential of improving the long-term quality of life of patients\(^1,2\).

Injection of suitable solution is used to separate the neoplasm from *muscularis propria*. If the lesion is clearly distinct visually, it usually means that there is no deep submucosal invasion. On the other hand, the “non-lifting sign” has been found to have 100% sensitivity, 99% specificity, and 83% positive predictive value for invasive carcinoma\(^3\).

Successful elevation of neoplasm allows the application of polypectomic loop and control of incision process. Composition of the injection solution for EMR use is not standardized\(^4\) but efforts have been made to improve coagulant and
colouring properties of such compositions. In literature, the use of coagulant as a highly concentrated salt solution with the addition of adrenalin or epinephrine is reported\(^5\). Alternatively, the physiological solution with addition of the derivatives of cellulose, succinyl gelatine, glycerol and fibrinogen have been formulated to slow down diffusion\(^4\). The physiological solution with addition of methylene blue or sodium salt of indigotindisulfonate was applied as a visual tool staining the neoplasm\(^5\).

The disadvantage of currently utilised solutions for diagnostics and surgical treatment of neoplasm of the GI tract is mainly short time of the elevation of the lesion (separation of adenoma) as well as only moderate visual distinction from surrounding tissue. The lifetime of such raised adenoma is determined by fast diffusion of injected solution, which leads to the disappearance of adenoma without its colour distinction. In the case where auxiliary colouring agent is used, the boundary between the adenoma and healthy tissue is dispersed due to rapid diffusion of the colouring agent into both volumes.

During our search for the optimal composition of solution, we have found the very pronounced effect when specific injection solution - composed from components approved for systemic administration and administered under valid law - was applied as submucosal injection into the neighbourhood of suspected neoplastic tissue as a part of EMR procedure. Several polyps raised above the injected tissue, lasting several minutes and forming pronounced stem allowing for comfortable application of polypectomic loop. The colouring compound formed three differently coloured volumes - injected tissue, neoplastic tissue and thin boundary between them, further helping to
diagnose the extent of neoplastic tissue and its level of embeddedness. The new empirically found composition clearly and repeatedly improved the EMR procedure when compared with actual clinical practice, but also when compared with literature.

In our attempt to elucidate the behaviour observed during EMR, we have performed the in vitro experiments on HCT116 (human colorectal carcinoma) cell line subjected to the conditions as present during endoscopy. In the paper we report our observations as well as the suggestion of the mechanism of action based on our limited experiments and known facts.

Materials and Methods

Preparation of EMR solution. The EMR solution we used is three-component: physiological (saline) solution, in which the visual contrasting aid-the sodium salt of [4-(alpha-(4-diethylaminophenyl)-5-hydroxy-2,4disulphophenyl-methylidene)-2,5cyclohexadiene-1-ylidene]diethyl-ammonium hydroxide inner salt (PATENTEBLAU V sol. inj. 2ml/50mg, GUERBET, France)\(^6\) and the colloid modulator of velocity - a Hydroxyethyl starch (HES) (VOLUVEN\(^\copyright\) sol. inf. 1x500ml, Fresenius Kabi, Bad Homburg, Germany)\(^7\) was dissolved. We prepared a mixture of EMR composition by mixing 500 ml of isotonic saline solution with 1 ml of colour constituent (PATENTEBLAU V) followed by diluting with a HES drawn up into the syringe with Combi-Stopper (syringe bung) in the ratio 3:7 under aseptic conditions and apyrogenic (dilution closed path) in a laminar flow hood.
The selection of suitable patients and administration protocol for the use of such a contrast mixture was limited by the estimated size of the adenoma in their large intestine larger than 20 mm.

The patients were subject of EMR polypectomy in Central Military Hospital SNP Ružomberok, Slovakia at gastro-intestinal clinic. In total, 50 patients were administered the composition during the period of three months starting from February 3rd, 2014. The procedure was approved by the Ethical committee of the Central Military Hospital SNP Ružomberok, Slovakia according to the valid Slovak law pursuant to §18a, No. 140/1998 Coll. of 3 April 1998 on Medicines and Medical Devices, on the Amendment of Act No. 455/1991 Coll. on Trade Licensing (Trade Licensing Act) as amended and on the amendment of the Act of the National Council of the Slovak Republic no. 220/1996 Coll. about advertising for the non-interventional clinical trials. Informed consent was obtained from all patients.

During the endoscopic session, the close neighbourhood of suspect tissues were injected by submucosal injection via endoscopic channel in 1-5 ml volume, leading to the bolus and subsequent volume and colour changes. The elevated polyps were removed using polypectomic loop and removed tissue analysed for histology. As a rule, several polyps became visible with formed stem lasting several minutes, so that they could be removed in one-step.

Cell line model. The human cancer cell line HCT116 was purchased from American Type Culture Collection (ATCC) and cultured in RPMI 1640 growth medium (Biosera, Kansas City, MO, United States). The growth medium was supplemented with a 10% foetal bovine serum (FBS),
1x HyClone™ Antibiotic/Antimycotic solution (GE Healthcare, Little Chalfont, UK) and maintained in an atmosphere containing 5% CO₂ in humidified air at 37 °C. Before experiments, the viability of cells was analyzed by trypan blue assay. For experiments, cells were seeded in 96-well low-density plates and maintained in a full culture medium for 24 hours. The cells were then starved in saline solution without nutrients for 24 hours, mimicking the preparation of patient before EMR surgery.

The initial cell culture was grown to 10 thousand cells per well, forming plaques. During the starving, part of the cells detached and floated freely in the medium. We removed free floating cells with the part of liquid media, removed volume was then restored by adding the same volume of the saline solution. Despite the fact that the saline solution detaches part of the starving cells, the significant fraction of the starving cells stays attached to the density plate after adding the saline solution.

*Adding the contrasting composition.* The EMR composition was applied for two groups of cells – nonstarving and after 24 hours starving in saline solution. After 24 hours, the non-starving cells were adherent to the density plate, while in starving cells, the free-floating cells had to be removed and media replenished by saline solution first. In both cases, the last step of the procedure consisted of replacing the saline medium by EMR composition. For starving cells about half of the volume of saline was replaced by EMR composition. Subsequently, we have prepared a set of the substance without a colour constituent. In all cases, the cells exposed to the solution were followed by 10 minutes of live video flow on a Cytation 3 Cell Imaging multimode
sensor (BioTek Instruments, Inc.) and evaluated visually for cell count, cell volume and cell shape change.

**Results**

*EMR use.* Under the conditions specified above, the EMR composition was applied for 50 endoscopies with adenoma qualifying for EMR. The typical reaction to administration of contrasting composition by submucosal injection on adenoma larger than 20 mm is depicted on Figs. 1 - 4. EMR composition provides colour contrasted differences between the tissues. The normal tissue is of light blue colour, the thin boundary between the adenoma and the healthy tissue is dark blue, while adenoma is not coloured. By injection of this EMR composition into submucosa layer, the adenomatous polyps increase noticeably their volume and elevate above the surface for the duration of 10-25 minutes, prolongating the time window for the resection. At the same time sharp colour differences between the healthy and the neoplastic tissue and the boundary between them can be observed. Colour distinction as well as the increased volume of the elevated polyp thus increases precision and quality of polypectomic surgery.

*In vitro model.* For non-starving cells, we observed no significant changes when saline solution was replaced by EMR composition. This is in sharp contrast with reaction of starving HCT116 cells. In repeated experiments, the HCT116 cells starved by 24 hours in saline solution detached completely from the density plate. On Fig. 5 and Fig. 6 we document rare case, where the group of detached cells remain partially attached, so they could be localised and recognised. In other cases, the reaction of cells was so pronounced, that
the cells completely disappeared from the visual field of microscope and were not identified. For the cells on both Fig. 5 and Fig. 6 we could identify the estimated cell volume changes about 4% but for the majority of experiments, the expected volume expansion leading to the separation of cells must be higher but was not quantified.

The experiments repeated after two weeks with different HCT116 culture confirmed the same results.

**Discussion**

Our original attempt to improve the EMR composition led us to observation of significant differences in the response exclusively for starving cells. Starvation of *in vitro* cell culture was achieved by leaving the cells for 24 hours in saline only (no standard fasting medium was used). In clinical practice, the starvation is achieved by the patient abstaining from oral food and fluid intake for 24 hours (*nill per os*) before EMR.

The only component of EMR composition capable of triggering such reaction is HES. The lower intestines are not involved in starch digestion so the differences must be due to starch processing capabilities of neoplastic tissue.

The administration of EMR composition into submucosa exposes the embedded part of the adenomatous polyp to the HES - chemically modified starch. HES as large macromolecule (average molecular weight 130 kDa)\(^9\), is expected to diffuse slowly in submucosa. Indeed, this can be seen by comparison of Fig. 2 and Fig. 3 separated in time by 150 seconds.
We expect to find alpha-amylases (or functionally equivalent enzymes) present in submucosa and released to the environment by starving neoplastic cells. While we found no direct data supporting the expression of starch-processing alpha-amylases in gastric adenomas, they are reported in similar and thus related lung adenocarcinomas\textsuperscript{10}. Under the conditions indicated for EMR the adenoma polyps of size exceeding 20 mm are still mostly benign – less than 10% are further progressed along known adenoma-carcinoma sequence\textsuperscript{11}. The probability of 50 EMR resections being all carcinoma is thus extremely unlikely and we shall assume, that already adenomas express alpha-amylases in sufficient amount. Due to the protocol the alpha-amylases are present even when part of the liquid (saline solution used for starving) is removed - in the time scale of experiments the \textit{de novo} synthesis of alpha-amylases should not manifest within tens of seconds of HCT116 exposition to EMR composition.

If alpha-amylases (or their functional equivalents) are present in submucosa, the initial volume of iso-oncotic HES can be degraded, and progressively smaller hydrolysis fragments formed in submucosa. In contrast to blood plasma, even the fragments below the renal threshold (45-60 kDa)\textsuperscript{12} remain available for further hydrolysis. As a result, continuous supply of glucose and hydroxyethyl glucose (hydroxyethylated at C2/C6 ratio 9.05:1)\textsuperscript{9} is delivered into submucosa. The dynamic mixture of fragments including the final monosaccharide product of alpha-amylases is produced in submucosa, contributing to rise of oncotic pressure.

Without transport of monosaccharides, the oncotic pressure would manifest as volume increase of submucosa. In our observation, the dominant volume changes are observed in the volume of neoplastic tissue. The volume change is also
observable in vitro at adherent 2D plaques formed by model HCT116 line. While carcinomas are known for enhanced expression of glucose transporters, adenomas must acquire the capability gradually. It seems thus reasonable, that polyps larger than 20 mm indicated for EMR possess enhanced amount of glucose transporters, particularly GLUT1\textsuperscript{13,14}.

In healthy tissue, the transport of monosaccharides proceeds in direction from lumen to serosa and the transport for excessive concentration of monosaccharides is facilitated. The adenomas expose the surface present to lumen also to the submucosa, so that the direction of facilitated transport of monosaccharides is reverted.

The facilitated transport of sugars into cells is specific in the sense, that different saccharides are transported with different efficiency. Also, the hydroxyethyl glucoses are expected to transport inside the cells less efficiently than anhydrous glucose. Thus, the depletion of the pool of oncotic pressure generating saccharides in the submucosa volume follows complex kinetics, well beyond our current focus.

Nevertheless, the in vitro HCT116 model cells eagerly transport oncotic molecules, as demonstrated by violent volume changes leading to loss of cell adherence. This kinetics can be the reason that the polyp rather than submucosa increases the volume in clinical observation/application.

EMR administration details. The EMR protocol which was applied corresponds to the submucosal injection of the EMR composition, where the actual composition is delivered into submucosa.

The resection starts by administration of the EMR composition below the adenoma - into the healthy submucosa. The preparation of patient includes 24 hours fasting.
HCT116 cell line forming 2D cell plaque is often utilised as an imperfect model of human colon cancer cells. The adenomatous polyps larger than 20 mm have increased probability of being malignant\textsuperscript{15} and thus HCT116 should be reasonably representative of the most numerous and voluminous cell composition of the expected stages of adenoma progression during reported EMR.
Conclusion

The modification of EMR composition and the application protocol lower the application barrier for EMR by improving the comfort and precision of the EMR. By clear delineation of the polyp boundary and the volume changes, lasting longer time, the polypectomy can be done in less time-related stress and lower risk of unwanted complications.

Our work indicates - somewhat surprisingly – that those adenomas in the early stage of transition adenoma-carcinoma already express alpha-amylases and exhibit elevated glucose transport responsible for volume changes. This provides the opportunity for functional diagnostics similar in spirit to fluorodeoxyglucose contrasting in PET².

The explanation we present suggest the possibility to modify the application protocol by taking advantage of different pharmacokinetics based on controlled and tuneable development of oncotic pressure. This can be probably used to develop more selective drug delivery to more specifically characterised target tissues.
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Figures

Fig. 1  Endoscopic findings before submucosal injection of the EMR composition. The size of the polyp is about 4.5 cm. (Time T+0 seconds immediately before submucosal injection)
Submucosal injection of EMR composition below the adenoma. The surrounding healthy tissue is of light blue colour, the adenoma colour is nearly unchanged. Note the rise of volume of the adenoma. (Time T+150 seconds after submucosal injection of EMR composition)
Fig. 3  Thin dark blue boundary is formed between the adenoma and surrounding healthy tissue. Polypectomic loop is inserted. Note the visible progress of diffusion of the colour in the healthy tissue. (Time T+300 seconds after submucosal injection of EMR composition)
Fig. 4  The resection and complete removal of the GI adenoma. The mucosa and light blue submucosa below are visible after resection. (Time+ 330 seconds after submucosal injection of EMR composition)
Fig. 5  Group of starving cells HCT 116 with free floating cells removed (bright field image)
Fig. 6. The same field of view as in Fig. 5 after the cells were exposed to combination saline solution and HES in a ratio 3:7. After T+30 seconds the cells detach and for most part float away from the field of view (bright field image)
Role of authors:

Patrik Jakabčin conceived and elaborated the idea, clarified the legislative conditions and prepared the EMR composition, performed the cell-line *in vitro* experiments, participated in writing paper. Martin Kello cultivated the cell-lines and performed the *in-vitro* experiments. Jozef Záň performed the EMR resections. Josef Kolář advised the clinical pharmacology aspects. Jozef Uličný conceptual design of the paper, supervising, assisted during *in-vitro* experiments, writing-review and editing.

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