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REVIEW ARTICLE

Intravesical Chemotherapy and Chemohyperthermia in Non-Muscle-Invasive Bladder Cancer; An Overview on Drug Administration Technologies and Pharmacokinetics

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Abstract: ***Background:*** Tumor recurrence is the most expected clinical event after the resection of non-muscle invasive bladder cancer, depending on histological findings of the initial lesion. In patients with low and intermediate risk of disease, the intravesical instillation of chemotherapy agents is recommended as a standard treatment to reduce recurrences.

Methods: A comprehensive review covering various aspects of different treatments with intravesical drugs is presented.

Results: Drugs may be instilled into the bladder starting with a single, 'early' postoperative administration or, after tumor resection with adjuvant intent or, before tumor resection under a neo-adjuvant regimen. Both latter protocols would consist of weekly treatments followed by monthly maintenance treatments. Different methods of administering drugs intravesically have been proposed to enhance the depth of drug penetration and its absorption into the bladder wall thus increasing the rate of drug-DNA reaction. These device-assisted therapies therefore have set a goal to potentiate the drug's effect and efficaciousness. The Radiofrequency-Induced Thermochemotherapeutic Effect (RITE) and the Electromotive-Drug Administration (EMDA) are the two most relevant modalities used to increase the activity of intravesical chemotherapy. Despite the widely adopted international guidelines' recommendations, and recent clinical trials of device-assisted chemotherapy instillations showing markedly enhanced recurrence-free survival compared even to the standard of care, clinicians and pharmacologists are not familiar with the in-depth physical aspects, pharmacokinetics and systemic absorption of chemotherapeutic drugs following their intravesical administration.

Conclusion: Knowledge of drug diffusion mechanisms into the tissue and cellular cytoplasm following bladder instillation is a key to understand the safety profile and clinical activity of chemotherapy.

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1. INTRODUCTION

After malignancies of the prostate, lung, and colon, the fourth of incidence is bladder cancer, but the high recurrence rate makes it probably the most prevalent malignancy, and the most expensive due to the number of treatments per patient [1]. There were an estimated 151.200 new cases of bladder cancer and 52.400 deaths from this disease in Europe in 2012. More than 90% of bladder cancers are urothelial cell carcinoma (UCC), and on average 70% of bladder UCCs present as non-muscle-invasive bladder cancer (NMIBC). As many as 80% of patients with pTa disease will experience disease recurrence, and up to 45% of patients with pT1 or CIS (carcinoma *in situ*) will experience disease progression without treatment [2]. The intravesical administration of drugs is the most intuitive approach to the urothelial surface of the bladder. Unfortunately, the permeability of mammalian bladder epithelium is low, thus the drug concentration and exposure time are paramount in determining tumor response to chemotherapeutic agents [4]. Intravesical chemotherapy and immunotherapy are nowadays the first choice adjuvant treatment for NMIBC. An immediate instillation after transurethral resection of bladder tumors is advised for all patients with apparently low-intermediate risk NMIBC, because it reduces the risk of recurrence by about 50% at 2 yr and >15% at 5 yr [5-7]. It acts by preventing tumor cell implantation and by an ablative effect on residual tumor cells at the resection site and on small overlooked tumors. In low-risk patients an immediate instillation is considered to be the standard and sufficient treatment. For other patients, however, an immediate instillation remains an incomplete treatment because of the considerable likelihood of recurrence and/or progression. A cycle of instillations with a chemotherapeutic drug is treatment of choice for patients with intermediate-risk NMIBC because it is able to reduce the short-term risk of recurrence. Mitomycin C (MMC) is the most widely used intravesical chemotherapeutic agent, other common drug is Epirubicin [8, 9]. Intravesical Bacillus Calmette-Guerin (BCG) is the most active intravesical therapy in intermediate-risk and high-risk patients [10, 11]. Drugs instilled into the bladder cavity will dilute to approximately 10% of the original administered concentration after one hour [12]. This is a result of urine production, exfoliation of particles and cells and the absorption of the drug. Drug concentration

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has top and bottom boundaries between which it is most effective; *e.g.* the lower limit for MMC is 0.12 mg/ml, and the upper limit is 0.25 mg/ml [13], where the MMC activity and induced apoptosis reaches 90% (beyond that limit, the activity is minimal but the risk of side effects increases). Also, like many other agents, MMC itself is a pro-drug and relatively unreactive towards DNA, and must undergo chemical alterations to bond with the DNA [14]. Since time, concentration, activation, together with other factors discussed in this article, govern an effective bladder instillation, ways of potentiating the treatment are constantly being investigated.

In the last decades new techniques of intravesical chemotherapy have been introduced, with special devices exploiting different principles in order to increase the effectiveness of adjuvant therapy (Thermochemotherapy [15, 16] and ElectroMotive Drug Administration [17]). In this review the principles of pharmacokinetics and absorption of chemotherapic agents administered intravesically for NMIBC are described, as well as the techniques to maximize drug delivery and contact time and strategies to enhance the absorption and action of these agents.

2. PHARMACOKINETICS AND ABSORPTION OF INTRAVESICAL CHEMOTHERAPEUTIC DRUGS

Studies on absorption and drug concentration into the bladder wall have carried out in dogs [18]. The bladder of the dog is about half in thickness compared to the same human organ. The microscopic anatomy of the bladder is similar among the two species, composed by the urothelium, basement membrane, connective tissue underlying including the capillar network, and muscle and serosal layers. The epithelium is structured in 3 to 5 cell layers, with tight junctions waterproofing the upper cell line. Beyond the urothelium the drug diffuses in the extracellular space with consequent intracellular passage. The concentration slope of the drug into the connective tissue is dependent on the tissue depth and the drug washing is determined by the capillary removal, as reported by Flessner *et al*. [19]. The bladder wall can be considered as a barrier (the urothelium) covering a layer composed by capillary perfused tissue. Across the urothelium the drug concentration drops suddendly; the overall bladder wall concentration declines linearly with respect to tissue depth. The drug diffuses through the deeper tissue, and is cleaned by the capillary flow.

Two models of drug concentration have been proposed in experimental settings. The diffusion model is based on the passage from a layer of high drug concentration (the urothelium) to a deeper layer with low concentration (muscular and serosal). In this model the concentration declines with depth, below the urothelium to the serosal layer.

In the distributed model the drug is removed by capillary drainage in addition to drug diffusion. In this model the concentration decreases following a log-linear slope. A plateau concentration is reached in the deep tissue where there is an equilibrium among the tissue concentration and capillary perfusion. The plateau level is reached at tissue depth beyond 2000 μm. the average bladder tissue concentration does not correlate with the time of instillation. In dogs plasma concentration of MMC increases rapidly and a plateu is reached within 10 to 30 minutes.

Wientjes M.G. *et al*. reported the penetration of MMC in dogs' bladders harvested after the intravescial instillation [19]. The drug concentration was 20 mg/40 ml as the commonly prescribed in humans. They found an interindividual variation of bladder tissue drug concentration among animals. This variation may be explained by the different bladder distension among dogs. However, after an intravesical dwell time of 5-7 minutes, the MMC concentrations in tissues were similar to those found after 30-120 minutes. The negative correlation of tissue concentration and duration of intravesical dwell time suggests that a steady state is rapidly achieved between MMC in urine and tissue. The plasma concentrations of MMC ranged from 2 to 94 ng/ml, which is at least 5000-fold lower than the average intravesical concentrations of 500 μg/ml, considering the variation of urine dilution. The plasma concentrations have a rapidly increasing slope with a plateau reached within 10 to 30 minutes after the intravesical instillation.

The same authors studied the tissue concentration of MMC in the human bladder [20]. A group of 24 patients underwent intravesical administration of MMC 20 mg/40 ml before the intervention of radical cystectomy, for muscle-invasive tumors. At last 1 hour after the MMC instillation the bladder vascular pedicles were ligated and bladders removed. Microscopic sections of the bladder wall were taken parallel to the urothelium and analyzed for MMC concentrations. In 13 patients tissue concentrations of MMC were not detectable with accuracy, since the highest concentrations were near 0.1 μg/g. In only 4 patients the MMC was detected at the upper urothelial layer. In 7 patients the bladder wall contained significant MMC concentrations, with a peak > 2 μg/g. Their tissue concentrations were correlated with depth; the urine concentration was 20-50 fold higher than the concentration in the lamina propria. After the drug penetration under the urothelium and basement membrane (200 μm), and lamina propria (700 μm), the muscle layer reached a relatively stable level of MMC, at a depth of 2000 to 4000 μm.

The mean plasma concentrations were one million-fold lower than urine concentrations and 10 to 1000-fold lower than the bladder tissue concentrations.

To obtain an effective intravesical chemotherapy is important to optimize the drug solution. MMC is stable at pH 6 [21, 22], Thiotepa and its active metabolite at alkaline pH (8.4) [23, 24], whereas Doxorubicin is more stable in acidic pH [25]. The antitumor activity is also affected by pH. MMC is more active at acidic pH in monolayer cultures [26], Epirubicin at alkaline pH (8.0) [27].

3. PHARMACOKINETICS OF CHEMOTHERAPEUTIC AGENTS ADMINISTERED AS EARLY-SINGLE INSTILLATION AFTER TURBT

TURBT is the standard approach for diagnosis and treatment of NMIBC, however up to 45% of patients will have a recurrence within 1 year after TURBT alone. This cancer behaviour can be related to the incomplete resection of primary tumor, the polychronotopic urothelial dysplasia preluding tumor development, the presence of undetected flat tumor and the potential cell seeding early after transurethral resection [28]. To date the main goal of reducing recurrence in patients with Ta-T1 NMIBC is obtained by intravesical chemotherapy, even if it does not prevent progression [29]. The European Association of Urology (EAU) guidelines recommend an early single instillation at TURBT in patients with NMIBC [30]. This alternative approach may decrease local and systemic toxicity, and cost, while maintaining the reduction of recurrence rate especially in low-grade tumors. The safety and absorption of intravesical chemotherapy has well been reported when the drug is administered in intact bladder for some time after TURBT, but there is a lack of studies investigating the systemic absorption when the drug is instilled immediately after TURBT. The most widely used chemotherapy in intravesical treatment of NMIBC is MMC. Gemcitabine (Gem) is the most recent drug which has been well studied in pre-clinical, experimental and clinical trials applied to urothelial bladder cancer [31-35]. The pharmacokinetic profiles of these two intravesical drugs administered immediately after TURBT has been reported [35, 36], including the differences in systemic absorption according to to the extent of bladder resection in NMIBC (depending on tumor volume).

The study population was composed by patients with a primary or recurrent NMIBC, eligible for a single, immediate, post TURBT intravesical instillation of Gem or MMC.

Patients were divided in two groups depending on the overall tumor volume. Up to a sum of 2 cm in tumor diameter, the extent of resection was considered small, while for a size greater than 2 cm the resection was considered large.

MMC (Sigma-Aldrich, Milan, Italy) was prepared as a solution of 40 mg of drug diluted into 50 ml of distilled water (0.8 mg/mL). Gem (Eli Lilly, Indianapolis, USA) was prepared in solution of 2000 mg of drug diluted in 50 ml of saline (40 mg/mL). At the end of the TURBT the bladder wall was meticulously examined paying attention for eventual bladder perforation that is considered the absolute contraindication to intravesical instillation. Then the bladder was emptied before the drug was instilled through a urethral catheter, which was kept inside the bladder for a dwelling time of one hour. All patients were operated under epidural anesthesia and received restricted intravenous hydration during the procedure with the aim to reduce the drug dilution by urine production.

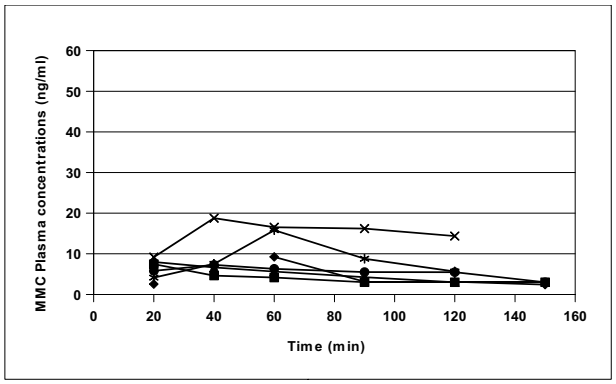
For MMC evaluation, blood samples were obtained at baseline, and at 20, 40, 60 (emptying time), 90, 120 and 150 minutes after the start of instillation.

For the evaluation of Gem and its inactive metabolite 2,2-difluore-2-deoxyuridine (dFdU), blood samples were collected at baseline, and at 15, 30, 60, 90 and 120 minutes after instillation. Each time 4 ml of blood sample were drawn, than centrifuged and freezed at -20°C for analysis.

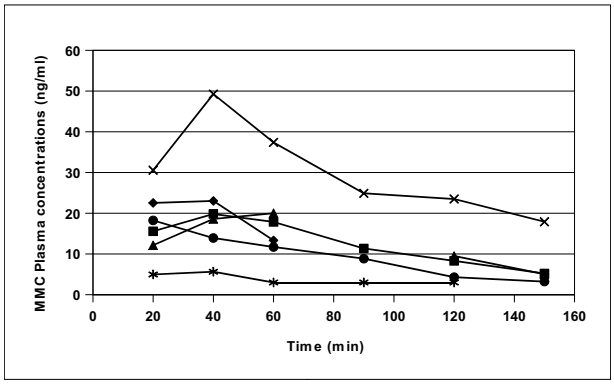
Concentrations of the drugs were determined by two different validated high performance liquid chromatography (HPLC) assays [37].

Twenty-nine patients were recruited and all of them successfully completed the study. Fourteen patients were allowed to receive intravesical treatment with MMC and fifteen others with Gem. Among the group instilled with MMC, 7 patients underwent small bladder resection and 7 underwent large resection; of the Gem group 7 underwent small resection and 8 a large resection.

Plasma concentrations of MMC and Gem in blood samples obtained at different time after intravesical administration are plotted in Figs. (**1** and **2**).

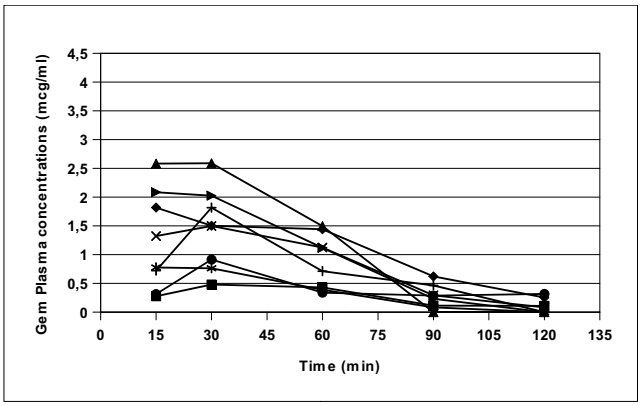


A

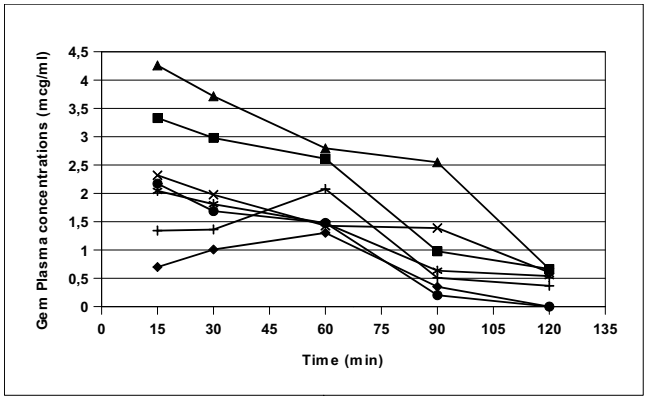


B

Fig. (1). Plasma concentration of MMC (ng/mL) after small (**A**) and large (**B**) TURBT.



A



B

Fig. (2). Plasma concentration of Gem (mcg/mL) after small (**A**) and large (**B**) TURBT.

The maximum MMC plasma concentrations were commonly reached at 40 minutes after instillation in 9 out of 14 patients, mostly in large TURBT (5 patients) and in all 12 evaluable patients within 60 minutes. At 150 minutes the minimal drug plasma level was only detectable in 4 patients who underwent large resection. The highest plasma peak was 49.25 ng/ml, far from reaching the value of 400 ng/ml associated with myelosuppression [36]. The systemic absorption of MMC was significantly proportional to the extent of the TURBT at the first plasma check, at 20 minutes ($p= 0.026$).

Plasma absorption of Gem was more rapid, reaching the maximum peak concentration at 15 minutes in 8 out of 15 patients (53%) and at 30 minutes in 13 (87%). Contrary to the MMC concentration slope, plasma level of Gem declined rapidly, even while being instilled into the bladder. The highest Gem concentration peak of 4.26 microg/ml was found in the large TURBT group; however it was consistently lower than the mean concentration obtained during the intravenous chemotherapy regimen (about 30 microg/ml)[35]. The comparison of plasma absorption between small and large bladder resection was significantly different at 15 minutes ($p<0.0001$) and maintained this trend until 1 hour ($p=0.05$). These studies demonstrated that the tumor volume and the related TURBT extent influences the systemic drug absorption when the urothelial barrier and the submucosal layer are removed endoscopically.

4. PRICIPLES OF INTRAVESICAL ELECTRO-OSMOTIC ADMINISTRATION OF CHEMOTHERAPEUTIC DRUGS

The principle of intravesical electromotive drug administration of MMC is a combination of several different electro-molecular interactions: iontophoresis, electro-osmosis/electrophoresis, and

electroporation are all responsible for electromotive transport of drug molecules in an electric field across biological membranes into underlying tissues [38]. Iontophoresis refers to accelerated ion transport into tissues by means of an electric current passed through a solution containing the ions to be administered at a rate defined by Faraday's Law [39]. Usually iontophoresis is associated with increased transport of water that will entrain any non-ionized solutes present, a phenomenon termed electro-osmosis. Electro-osmosis/electrophoresis is used to describe the current induced convective flow of water in association with ions, which can accelerate the transport of ionized molecules down coulombic gradients, non-ionized polar molecules [40], and ionized molecules against their coulombic gradients [41]. Electroporation implies increasing biological membrane permeability under the influence of an electric field, which increases transport rates down concentration gradients [42]. Drug transport rate is the algebraic sum of that induced by passive diffusion and by electromotive drug administration, but, when dealing with a membrane of low permeability such as the urothelium, electromotive drug administration is so dominant that it may be considered as the sole force manipulating drug transport. The administration rates are markedly increased and they are controllable by varying the current intensity. MMC is almost non-ionized within the full pH range tolerate by the bladder: 4.5-8.5 units [43]; but some preparations contain NaCl as an excipient. When these vials are dissolved in water, the NaCl conducts current, and the polar, non-ionized MMC is administered by electro-osmosis. Therefore, realistic clinical or laboratory investigations involve mainly the electro-osmosis [44] and probably electroporation [42]. The requisite ions for this mode of electromotive drug administration come as a sodium chloride excipient in the MMC crystals supplied. When a current of positive polarity is applied to a solution containing Na^+/Cl^-/MMC, sodium ions are iontophoresed into underlying tissues, a process that includes transport of water in the form of hydration shells around Na^+ [45] as well as in the free form [39]. Solubilized, non-ionized MMC is entrained in this water flux in quantities proportional to its concentration in the solution.

MMC penetration of the bladder wall has been investigated in laboratory in a two-cell diffusion chamber model [45]. Tissue sections of human bladder were inserted into two chamber cells with urothelium exposed to donor compartments containing MMC (10mg in 100 ml of 0.24% NaCl solution) and an anode with serosa exposed to receptor compartments containing 100 ml of 0.9% NaCl solution and a cathode [46]. Fourteen paired experiments (current 5 mA/no current) were conducted over 15 mm. This initial study showed that in all tissue samples exposed to electric current MMC concentrations were higher than after passive diffusion (21.5 ± 8.4 μg/g *vs* 3.4 ± 1.9 μg/g, p=0.0010), the variability in drug delivery rate was reduced (39.1% *vs* 56.7%, p=0.0010) and the electric current caused no histological tissue damage and no chemical modification to MMC.

In a second study MMC concentration-depth profiles in the bladder wall after passive diffusion and electro-osmotic administration were compared [44]. During each paired experiment, two-tissue samples from a single patient were placed between the two chambers of individual diffusion cells.

MMC (80 mg) and 1920 mg of NaCl were dissolved in 200 ml of bidistilled H_2O, and then the solution was divided into two volumes of 100 ml each (40 mg of MMC in NaCl 0.96% solution), which were placed in paired passive diffusion and electro-osmotic administration donor compartments. The receptor compartments were filled with 100 ml NaCl solution 0.9%. In electro-osmotic administration experiments an anode was placed in the donor compartment and a cathode in the receptor compartment. The electrodes were connected to the current generator and experiments were performed with pulsed direct current of 20 mA for a duration of 30 min. No electric current was applied in passive diffusion control experiments. Each tissue segment was rapidly weighed and frozen by placement of the urothelial side on a flat stainless-steel plate cooled on liquid nitrogen. The frozen tissue was glued onto the cryotome object holder and the urothelial surface was exposed for sectioning. The first 80 μm of tissue directly in contact with the MMC solution was trimmed off to avoid contamination by the dosing solution. Tissue was sectioned into 40-μm thicknesses, placed and stored frozen. For tissues between 80 and 200 μm in depth (urothelium), each 40-μm segment was analyzed individually. For tissue between 200 and 1200 μm in depth (lamina propria) and beyond 1200 μm in depth (muscularis), five and ten segments, respectively, were pooled for analysis. Bladder wall sections were analyzed by high-performance liquid chromatography for MMC concentration. Tissue viability and morphology and MMC stability were assessed by trypan-blue exclusion test, histological examination, and mass spectrometry analysis. MMC concentrations reached a plateau in all compartments after 15 min with no further increase during the 60 min of incubation. The mean concentration of MMC transported into the bladder wall by electro-osmotic administration (28.2 ± 4.0 μg/g) significantly exceeded the concentration achieved by passive diffusion (5.3 ± 0.8 mg/g), which is mirrored by the respective concentrations observed in the urothelium (170.0 ± 19.6 *vs* 46.6 ± 8.6 μg/g), lamina propria (65.6 ± 5.1 *vs* 16.1 ± 2.6 μg/g), and superficial muscle layers (15.9 ± 3.5 *vs* 1.9 ± 0.4 μg/g). The variability of MMC delivery was markedly reduced with electro-osmotic administration as compared with passive diffusion in all except the superficial muscle layer. It was concluded that electro-osmotic administration significantly enhanced MMC administration and reduced variability in drug delivery rate in all bladder wall layers, reaching IC90 levels [47-49] in the urothelium and lamina propria.

Much more convincing are *in vivo* data from a clinical study that compared intravesical MMC administered by passive diffusion or electro-osmotic administration in high-risk non-muscle invasive bladder cancer [50]. Following transurethral resection and multiple biopsies 108 patients with multifocal CIS were randomized into 3 equal groups of 36 each who underwent intravesical 40 mg electro-osmotic MMC instillation with 20 mA electric current for 30 minutes, 40 mg passive MMC with a dwell time of 60 minutes or 81 mg BCG with a dwell time of 120 minutes. Patients were scheduled for an initial 6 weekly treatments, a further 6 weekly treatment for non-responders and a followup 10 monthly treatment for responders. Primary end points were the complete response rate at 3 and 6 months. A total of 15 patients in the intravesical passive and electro-osmotic MMC arms, respectively, underwent pharmacological evaluation during the first intravesical instillation. Blood samples of 10 ml were drawn before and at certain time points after MMC instillation, including 0, 5, 15, 30, 45, 60, 90, 120, 180, 300 and 480 minutes. In both arms, passive and electro-osmotic MMC, bladder content samples were obtained at 5, 15, 30, 45 and 60, and 5, 15 and 30 minutes, respectively. Peak plasma MMC concentrations were significantly higher with electro-osmotic administration than passive diffusion (42.9 ± 7.1 *vs*. 7.8 ± 1.7 ng/ml). These data were consistent with bladder content absorption: for passive diffusion the absorption process was almost complete within 15 minutes and at 60 minutes approximately 50% of the initial MMC content was absorbed; with electro-osmotic administration at 30 minutes more than 80% of the initial MMC content absorbed. Although intravesical electro-osmotic administration markedly increased drug transport into the bladder wall, MMC plasma levels remained well below toxic concentrations (400 ng/mL). Side effects were significantly more prominent in the BCG arm than in the two MMC arms. There were no statistical differences between the 2 MMC arms, although there was a trend toward increasing numbers and side effect severity in the electro-osmotic MMC group. The complete response for intravesical electro-osmotic *vs*. intravesical passive diffusion MMC at 3 and 6 months was 53% *vs* 28% (p=0.036) and 58% *vs* 31% (p=0.012). For intravesical BCG the responses were 56% and 64%. Clinical response rates validated the prediction that

intravesical electro-osmotic enhancement of MMC delivery into high risk non-muscle invasive bladder cancers would provide results superior to those achieved using intravesical passive diffusion MMC.

5. THE RITE MECHANISM (RADIOFREQUENCY-INDUCED THERMOCHEMOTHERAPEUTIC EFFECT) IN TREATING BLADDER CANCER

5.1. Hyperthermia

The idea of inducing hyperthermia to selectively treat cancerous tissue has been well documented in various disciplines [51]. The accepted heating range is 42°C±2°C. The effective hyperthermic temperatures achieved would also harm cancer cells selectively, as their proteins unfold, heat shock proteins are formed, and ability to self-repair is inhibited at lower hyperthermic temperatures compared to healthy cells, and furthermore, the rate of reaction (drug-DNA bonding) is increased. Surprisingly, when treating four different bladder cancer cell lines *in vitro* (cultures were sunk in a heated bath), hyperthermia alone, was deemed an unsuccessful therapy since the surviving population of cells was over 84.7% in all 4 cell lines [52]. However, when combining heat with drug, a synergistic effect was evident. This was also proven clinically: Recurrence-Free Survival (RFS) was significantly improved compared to the drug alone (53% versus 15% in a 10-year follow-up on an RCT, $p<0.001$) as reported by Colombo *et al*. [53]. In that clinical trial, a dedicated device was used (Synergo® SB-TS 101, Medical Enterprises Europe B.V., The Netherlands), generating 915MHz Radiofrequency (RF) electromagnetic waves in conjunction with the instillation of a cytotoxic solution, and was described extensively previously [54, 55]. It employs the RITE (Radiofrequency-Induced ThermoChemotherapeutic Effect) mode of action, combining the simultaneous tri-modality of local, non-ionizing RF radiation, a constantly cooled chemo instillation and tissue hyperthermia achieved by the RF. Briefly, it consists of a computerized console and a specially-designated catheter incorporating an antenna and five thermocouples monitoring in real-time the urothelial temperatures of the bladder tissue, and urethral temperatures ensuring patient comfort on the urethra and drug stability, as the latter is known to reduce its activity when heated (MMC degrades by 15±1.2% in 30 minutes at 45°C at urine pH 5.5 [56]).

5.2. How RITE and Conductive Heating Differ

Previously it was believed that the clinical results in treating NMIBC achieved with RITE were accounted for by the conjunction of hyperthermia with chemotherapy. However, a recent study by Ware *et al.* [57], conducted on pancreatic cell carcinoma aside healthy cells with RF radiation, suggested that the RF plays a major role, (the maximal temperature reached in their experiments was only 38.68°C). The authors mentioned that compared to the healthy cells, cancer cells phenotype; motility, morphology, topography and division, changed with RF. They demonstrated how the adhesion between cancer cells was lost. From this we may infer that transport of drug molecules to and within deeper layers would be facilitated. They summarized that an RF treatment may have consequences on diffusion of anticancer drugs, cancer cell susceptibility and that various sub-populations heterogeneously respond to the treatment. Most interestingly, they reported the formation of TNTs ('Tunneling NanoTubes' or micropores) on the cellular membranes of cancer cells, which enable the penetration of molecules into these cells. The electric potentials created with the Synergo® device are of the same magnitude as in the mentioned article thus inevitably driving to the conclusion that the clinical results obtained thus far with the RITE method, certainly may *not* be attributed to hyperthermia alone. The authors also pointed out that a faster diffusion rate was achieved. This is accounted for by the generated 'Foucault' electrical currents produced by RF radiation that are responsible for charging the molecules of the drug and mobilizing them. Fick's first law determines that the diffusion rate would normally be:

$$J = \_\, D\frac{\partial C}{\partial x}$$

(Where J is the flux of the diffusing component [amount of particles passing through a plane in any given second], D is the diffusion coefficient and $\partial C/\partial \chi$ is the gradient [concentration/distance]).

When the Foucault currents are applied, the equation changes to:

$$\frac{1}{D}\frac{\partial C}{\partial t} = \nabla\left(\nabla C + \frac{CZ}{kT}\nabla\varphi\right)$$

(Where t is time, ∇ is the del or gradient operator, Z the valence of the charged particles, k the Boltzmann constant, T is the absolute temperature [in Kelvin degrees] and φ stands for a single charged particle moving in the electrical field).

This active diffusion was not reproducible with a drug that was heated [58]. In addition to all this, the RITE tri-modality induces a direct thermal effect in tissue - from an oncologic point of view, a welcomed by-product of RF radiation. The tissue temperature rise is uniform over the thickness of the bladder since the radiation profile of the one half-wavelength dipole antenna was designed to conform to the shape of the bladder.

Curley *et al.* [59] speculated whether all biological effects of RF treatment are limited to its hyperthermic property. The study they conducted compared how RF (without causing hyperthermia) and conductive hyperthermia treatments change the proliferation rate, oxygen consumption and autophagy in malignant and nonmalignant cells. They discovered that only RF treatment caused declines in cancer cell viability and proliferation. In their experiments, RF treatment also affected mitochondrial function in cancer cells more than the conductive hyperthermia treatment did and, unlike the latter; RF treatment was followed by the elevation of autophagosomes in the cytoplasm of cancer cells. Importantly, the effects of RF treatment were negligible in nonmalignant cells, they reported. More differences between RITE and conductive heating are mentioned in the following sections.

5.3. History of RITE

RITE was used to achieve hyperthermia of the bladder *via* RF radiation since the early 90s. Even if one were to disregard the RF as the main aspect in the plethora of articles on RITE so far published, and instead focus on hyperthermia alone with concurrent chemo instillation, it is imperative to investigate whether the heating of the instilled drug would in fact warm the tissue. An in-vivo experiment conducted in 1975 [60] demonstrated that when bladders were instilled with liquid at a temperature of up to 60°C for as long as two hours, only patches of necrosis were formed, from which we may infer that denaturation of proteins did not take place, implying that the temperature at the first layer of the urothelium barely reached 45°C. The reasons for this are several, both physical and physiological. The heated liquid must at all times be hotter than the target in order to transfer energy. Even though this was clearly the case in this experiment, the rich vasculature [61] of the lamina propria vasodilates and helps to effectively dissipate the heat. Moreover, the conductive thermal coefficient of the mucosal membrane composing the urothelium is low (roughly 0.34 W/m/°C) [62], meaning it is a good thermal insulator, thus making its conductive heating very inefficient.

In fluid dynamics there is a distinction between two kinds of flows; turbulent and laminar. Turbulent could be described as uncontrollable (it mixes and fluctuates in magnitude and direction). Laminar flow is the ordered parallel stratification of 'inner' flows according to their vector velocity and applies to the liquid flow on

the bladder walls. Liquid flow is known to show zero velocity near the banks or circumference. As the liquid radially approaches the bladder walls and as it glides over the urothelium, its speed decreases to nil. That would mean an additional layer of insulation is added on top of the urothelium.

Another notable difference: the quick drop in temperature with conductive heat (catheter compared to tissue) creates a barrier of a relatively cold cytotoxic drug on the target surface, yet another blocking of the conductive molecular vibration, and by so the diffusion of molecules into deeper layers.

Since energy transfer is determined by physical constants and thermal cooling by the blood, an increase of flow will only decrease the temperature difference between the inlet and outlet on the catheter and leave a similar amount of energy behind in the bladder. Using RITE enables the increase in power and thus the increase in energy transfer. This can be used to overcome the increased cooling by the blood bed due to vasodilation.

An experiment with heated MMC [63] showed that in at least half the subjects, the amount of MMC retrieved at the end of the experiment (after two 22 minute-cycles of instillations were administered) was higher than the amount placed in the bladder after the first cycle, with a median of 99.6% of the drug recovered. One plausible explanation of this phenomenon is the aforementioned barrier of drug created on the urothelium, only to be retrieved at the end of the treatment (substantiated elsewhere [58]). Retrieved concentrations *should* have been comparable between the two consecutive cycles to ensure continuous and homogenous absorption into the urothelium, similar to those with a body-temperature chemotherapy (0.16 ± 0.05 and 0.17 ± 0.05 mg/ml) and with RF radiation (0.12 ± 0.04 and 0.13 ± 0.03 mg/ml) [51] (with the second cycle usually being retrieved at a slightly higher concentration since the tissue is more saturated). The above clearly demonstrates that heating the cytotoxic drug and heating the tissue by means of electromagnetic radiation employ two very distinct pharmacokinetic mechanisms. The conclusion withdrawn from that in-vivo trial was that a second cycle of instillation was futile, as no further migration of MMC molecules into the tissue was evidenced. With RF, even deeper layers beyond the blood vascularization layers obtain elevated temperatures. Deeper heating ability allows MMC drugs to perfuse and diffuse into the tissue with the possibility to have constant introduction of 'fresh' molecules into the field.

Upon experimentation of the use of heated drug (MMC), researchers concluded that the "suboptimal heating profiles" may be different than with RF [64], the absorption rate was negligible [63] and these are part of the reasons for the different clinical results. Another clinical paper [65] comparing the use of a heated MMC solution to the use of BCG, concluded that the Recurrence Free Survival (RFS) was significantly improved with the administration of BCG. In comparison, the recent paper on a multicentric, Randomized Controlled Trial of RITE *vs*. BCG in first line [66] showed a statistically significant superiority of RF+MMC over BCG in terms of RFS (81.8% *vs*. 64.8% in the PP, p=0.02). Though it has its limitations of an indirect comparison, it is clear that the mechanism employed in heating the drug, also in its clinical results, differs greatly from the effective RF mechanism.

5.4. Pharmacokinetics in RITE and "Dose Splitting"

Paroni *et al.* [56] assessed the effect of local hyperthermia on the systemic absorption of MMC during intravesical chemotherapy. The population was divided into four groups receiving different doses (2×20 and 2×40 mg) both with and without RF-induced hyperthermia. The instillation time was 60 minutes with replenishment of solution after the first 30 minutes. The study demonstrated that the systemic exposure to MMC appeared significantly related to the drug concentration at the target site (20 *vs*. 40 mg) only when hyperthermia was associated with chemotherapy instillation, but not when chemotherapy alone was administered. These results demonstrate a statistically significant increase in the tissue permeability to MMC (up to 400%) due to the combination of RF and hyperthermia. Despite the significantly higher systemic concentrations of MMC during this device-assisted intravesical instillation, the systemic MMC levels are still far below the systemic toxicity threshold of 400 ng/ml for MMC, both during conventional intravesical instillation and during radiofrequency-induced hyperthermia treatment. The study demonstrated that the highest MMC plasma concentration (67.9 ng/ml) occurred after 45 minutes employing 40 mg of MMC, a 6-fold below the toxic threshold concentration.

Besides supporting its safety profile, these findings also indicate the clinical efficacy of RITE, as the markedly enhanced bladder wall permeability results in higher MMC penetration into the urothelium and the tissue layers, and the plasmatic levels only affirm this.

The dose administered in RITE treatments consists of two subsequent 30-minute dwell periods of 20 mg MMC each. These actually represent a total dose of more than 20 mg for the 60 minutes treatment. When administering MMC in two 30-minute cycles of 20 mg/50 ml aqua, as opposed to one dose of 40 mg/50 ml for one consecutive hour, there is a 10% additional exposure time of the cells (44 *vs*. 40 minutes, respectively) to the clinically-beneficial limits (0.12-0.25 mg/ml as discussed at the beginning of this section) - an adequate concentration when used with RF for most patients treated with prophylactic intent. The same applies for two 30-minute cycles of 40 mg/50 ml instead of one dose of 80 mg/50 ml administered for one hour, in patients with active tumors treated with curative intent (where the effective concentration of the drug must reach deeper locations in the tissue against the dropping gradient).

Another advantage of the "dose splitting" regimen used with the RF treatment it that it decreases the dilution of the drug with urine and thereby maintains a more optimal and homogenous drug dose throughout the entire treatment duration. This technique also minimizes the inter-patient variability and increases patients' tolerability, and has been used throughout the years with RITE [54-56, 66-71].

One more interesting discovery pertinent to RITE is the apparent immune response following the treatments. Significantly higher levels of MCP-1 and IL-6 and a borderline significant concentration difference of IL-8 and RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted) were observed in RITE-treated patients compared to body-temperature MMC instillations [67]. Most patients treated with the RITE showed decrease in proliferation activity and P53 activity - an effect not seen in patients treated with regular MMC instillation [68].

5.5. Acceleration of Rate of Reaction

As evident by the thermocouples which are deployed onto the bladder walls, in every RITE treatment, there is a rise in tissue temperature. The Arrhenius equation is a formula expressing the dependence of reaction rates on temperature:

$$k = A \times e^{-Ea/kBT}$$

(Where k is the rate constant, T is the absolute temperature (in degrees Kelvin), A is the pre-exponential factor, E_a is the activation energy for the reaction (in Joules), k_B is the Boltzmann constant).

For: T = 309 K (37°C), $k = 3.6 \times 10^{-10} \times sec^{-1}$

For: T = 315 K (43°C), $k = 8.2 \times 10^{-10} \times sec^{-1}$

Since there are 6 reactions (between the 7 stages as presented in the updated MMC's Iyer-Szybalski mechanism [14]) needing to take place for the MMC molecule to bond with the DNA, a tissue temperature rise to 43°C would theoretically accelerate this rate by almost 140 times compared to 37°C, since each of these reactions

would be taking place at the higher temperature: $(8.2/3.6)^6$ = 139.65.

5.6. Clinical Results

These mechanisms might well explain the Complete Response rates in patients with CIS (pure and concomitant), where the deeper basal-lamina layer and muscle layer interaction is of major concern: Witjes *et al.* [68] presented their data showing a 92% CR after the 8 weeks of induction. Evaluation of a large cohort of 111 patients, who had BCG-refractory high-risk bladder cancer, had an estimated disease-free survival of 85% and 56%, after 1 and 2 years, respectively [69]. In challenging the gold standard of radical cystectomy (RC), between 2006 and 2013, 96 patients who could not undergo or refused RC, were prospectively matched 2:1 with 47 patients who underwent the surgery [70]. This was a well selected group of patients, specifically BCG failure high-risk NMIBC. Whilst having similar baseline characteristics, and despite the RITE arm having a considerably higher Charlson Comorbidity Index score compared to the RC arm (6.1 *vs*. 4.3, respectively), there were no deaths associated with RITE compared to a ninety-day mortality of 4% in those receiving RC and the Disease-Specific Survival at five years was notably higher at 85.2% (RITE) and 74.6% (RC). The median follow-up was 36 months (3 to 88 months) for both cohorts. The authors report how the administration of RITE in selected high risk NMIBC patients does not expose them to a higher risk of progression during the course of the treatment, concluding that RITE provides durable long-term outcomes compared to RC for high risk NMIBC bearing a clear advantage in complication rates favoring it over RC.

5.7. RF Applications in the Future

RF-potentiation is not restricted to MMC alone, but also to other anti-neoplastic agents such as epirubicin [71]. As mentioned earlier [66], the statistically significant superiority of RITE over BCG could possibly lead to a paradigm shift in the treatment of NMIBC, as no treatment was found comparable to the BCG gold standard for 40 years. Having discovered that RF is likely to have been the main protagonist of RITE over the years [57] (as it also induces the hyperthermia), it is interesting to point out that the effects of RF treatment are specific to cancer cells and are not limited to its hyperthermic property [59]. As more prospective studies shed light and consolidate these findings perhaps new indications will be assessed for RF.

CONCLUSION

The peculiar structure of the bladder wall, based on umbrella cells interconnected by tight junctions, creates a barrier between urine and plasma. However, the urothelium is not completely impermeable and a quote of passive permeability is possible through transcellular and paracellular pathways. Some drugs administered intravesically demonstrated to be active against tumoral cells originated from the superficial layers of the mucosa. The instillation can be administered early postoperatively as a one shot treatment after tumor resection or as an adjuvant treatment. The corresponding systemic absorption has been investigated in order to optimize the most active intravesical concentration, taking into account the potential side effects. Device-assisted systems have been developed to enhance the biological effects of chemotherapeutic agents against bladder cancer cells. Intravesical electro-osmosis involves the application of electrical current across a biological barrier to accelerate the passage of drug through the urothelium. RF-induced chemohyperthermia in a recent randomized trial showed to be a promising treatment approach alternative to BCG immunotherapy. To date however, the regimen of treatment, including induction and maintenance, has not reached the definition of a standard schedule. Thus, further studies are needed to achieve a more solid clinical evidence [72]. Moreover, the knowledge of pharmacodynamics after the intravesical drug administration is crucial to understand the drug diffusion through bladder tissue and improve the development of novel drug delivery technologies [73].

CONFLICT OF INTEREST

G. M. Lev discloses being VP of Medical Enterprises Europe B.V., devisors of Synergo®.

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