Gastrointestinal Tract

ORIGINAL ARTICLE

Genotoxicity and safety profile of Eleview®: A submucosal injectable composition for endoscopic mucosal resection and endoscopic submucosal dissection

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ABSTRACT

Background and Objectives: Eleview® submucosal injectable composition with methylene blue (MB), is intended for use in gastrointestinal endoscopic procedures. Given the recognised *in vitro* mutagenicity of MB, Eleview® mutagenic and genotoxic potentials and its safety profile in acute and subacute toxicity settings were assessed. **Methods**: Acute and subacute systemic toxicity were tested in rats and dogs, respectively. Ames test and chromosome aberration test in Chinese Hamster V79 cells, were performed. The ratio of polychromatic to normochromatic erythrocytes was assessed in rat bone marrow at the rat MTD. **Results**: Eleview® oral or intraperitoneal at 20 mL/kg or 50 mL/kg did not induce any acute toxic effects in rats. In dogs, Eleview® (15 mL) did not cause any relevant in-life observations or any histopathological changes in any organs/tissues or injection sites. Ames test demonstrated no concentration-related and reproducible increases in revertant colony numbers. No significant increase of chromosomally aberrant cells was noted in Chinese Hamster cells. Lastly, Eleview® did not elicit significant increase in micronucleated polychromatic erythrocyte frequency. **Conclusion**: No death or abnormal findings in the acute and subacute studies were observed for Eleview® administration. Eleview® is not genotoxic, nor mutagenic, proving to be a safe medical device to use in clinical practice.

Key words: Eleview®, endoscopic submucosal dissection, endoscopic mucosal resection, polypectomy, methylene blue, genotoxicity, mutagenicity

INTRODUCTION

Endoscopic resection with different techniques such as polypectomy, endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD) have provided new alternatives for minimally invasive removal of gastrointestinal adenomas and early-stage cancers that involve a minimum risk of lymph-node metastasis.[1]

Submucosal injection is considered to play an important role in the EMR procedure, and the ideal injection solution should be both long-lasting and produce a hemispheric shape to facilitate snaring. In addition, it should provide a sufficiently high submucosal elevation for safe submucosal resection during the procedure. Normal saline (NS) is commonly used for this purpose. However, because of the rapid absorption of NS into

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the surrounding tissue, it is difficult to produce the proper submucosal fluid cushion and maintain the desired height.[2]

Cosmo Technologies developed the Eleview® submucosal injection solution that overcomes NS limitations for EMR, ESD and polypectomy procedures in the gastrointestinal tract. Eleview® is a medical device which is currently approved in several countries. The product contains a biocompatible polymer (poloxamer 188) which acts as bulking (cushioning) agent; it contains methylene blue as a dye; it is in form of a liquid, non-viscous microemulsion as this facilitates the injection of the product through a standard injection needle.

In US and in EU it is sold as single use plastic vials, 10 mL each. The pack contains 5 vials (https://www.medtronic.com/covidien/en-us/products/therapeutic-endoscopy/eleview-submucosal-injectable-composition.html). In the US, an additional product presentation (5 mL vials) is also commercially available (but not in other markets)

When injected, Eleview® creates a cushion *in situ* by lifting the gastrointestinal mucosa from the submucosal layer, allowing an easy and safe resection procedure. The vital dye methylene blue contained in the formulation allows a better identification of the tissues requiring resection. Use of methylene blue as staining dye for submucosal injection composition is widely described in specialized scientific literature and in endoscopic guidelines; this dye is more stable than indigo carmine, another common dye used in submucosal injection compositions. The content of methylene blue is extremely low. At the maximum recommended volume for use (50 mL), the product delivers 0.5 mg of methylene blue. For a 60-kg person, this equates to 0.0083 mg/kg.

Eleview® was tested in an *in vivo* porcine model showing no signs of adverse local or distant tissue reactions.[3] Additionally, in a double-blind randomized clinical study, comparing Eleview® to NS in EMR of colorectal polyps larger than 2 cm, Eleview® appeared to be more effective than, and as safe and as easy-to-use as, NS solution.[4] Methylene blue is a dye commonly used in chromoendoscopy.[5–7] Recently concerns were raised by a Regulatory agency that methylene blue might exert genotoxic effects when used in chromoendoscopy despite the fact that is routinely and safely used in humans.[8,9] As such, this specific study was conducted to address this concern.

The objective of this study was to assess the safety of Eleview® through the evaluation of toxicity (acute and subacute) and mutagenic and genotoxic potential in rats and dogs. Mutagenicity was measured using an Ames test and genotoxicity was evaluated in an *in vitro* chromosome aberration assay in Chinese Hamster V79 cells and in an *in vivo* murine micronucleus test.

METHODS

All tests were performed according to Good Laboratory Practice (GLP) regulation (Organisation for Economic Co-operation and Development (OECD) Principles GLP [C(97) 186 (Final)], except for the MTD test that was non-GLP. All tests were performed using commercial Eleview® batch WH047.

Acute systemic toxicity in the rat

The study was conducted to determine the potential acute systemic toxicity of Eleview®.[10] The test was performed in five male and five female Sprague Dawley rats with either a single intraperitoneal (IP) administration of Eleview® at 20 mL/kg or as a single oral administration at 50 mL/kg.

Two control groups of 5 animals received water for injection according to the same route and volume of the concurrent Eleview®-treated groups. On the day of dosing clinical signs were recorded pre-dose, 2, 4 and 6 h after dosing. Animals were observed daily up to 72 h after dosing, when they were subjected to necropsy and gross examination.

Subacute systemic toxicity (28 days) in dogs

The subacute toxicity of Eleview® was determined in Beagle dogs, administered *via* single submucosal injection in the esophagus, stomach and colon wall by endoscopic implantation in compliance with the current guidelines.[10,11] After treatment, dogs were followed for 28 days. On Day 1 endoscopic injection of 5 mL of Eleview® or water was performed in each animal group (3 animals per sex each) in the submucosa of esophagus, stomach and colon (15 mL/animal). Animals were observed daily for mortality, clinical signs, food consumption and body weight. The clinical pathology evaluation of hematology, coagulation, serum chemistry and urinalysis, was performed both pre-test and on day 29. Also, post-mortem necropsy, collection of selected organs/tissues and histological examination were performed.

Bacterial reverse mutation study (Ames assay)

Eleview® was evaluated for its potential to induce reversion mutations in *Salmonella typhimurium* tester strains TA98, TA100, TA1537, TA1535 and in *Escherichia coli* strain WP2 uvrA. This study was performed according to recognized international guidelines.[12–14] The mutation test was carried out using the preincubation method (30 min at 37°C) with and

without phenobarbital-5, 6-benzoflavone as inducer of rat liver metabolic (S9) activation. In the main assay Eleview® was tested with the preincubation method at concentrations from 1 to 5 μL/plate with and without metabolic activation, TA98 and TA1537, respectively. Conversely, in TA100, TA1535 and WP2 uvrA, only the concentration of 5 μL/plate was tested because, for soluble non-cytotoxic medical devices (determined in the range finding assay), a single test at one dose level is considered acceptable. Sterile water was used as negative control. For each strain, proper positive controls (Table 1A and 1B) were used to confirm the sensitivity of the test system to detect genotoxic damages.

In vitro chromosome aberration assay

To investigate the potential of Eleview® to induce structural chromosome aberrations in Chinese Hamster V79 cells, an *in vitro* chromosome aberration assay was carried out, in accordance with internationally accepted guidelines and recommendations.[14–19] The metaphases of Chinese Hamster V79 cells were prepared 21 h after start of treatment with Eleview®. The treatment interval was 4 h without and with metabolic activation in experiment I. In experiment II, the treatment interval was 21 h without metabolic activation. Duplicate cultures were treated at each concentration. 150 metaphases per culture were scored for structural chromosomal aberrations. Eleview® concentrations, 1.0, 1.5 and 2.0 μL/mL, were evaluated by microscopic analysis for both experiments. Ethyl methanesulfonate (EMS) at the concentration of 600 μg/mL was used as positive control in the experiments without metabolic activation while cyclophosphamide (CPA) at the concentration of 0.83 μg/mL was used as positive control in the experiments with metabolic activation. Negative controls were included. For each concentration, 300 cells were evaluated, except for the positive control with metabolic activation (CPA: 130 cells).

Maximum tolerated dose

A toxicity study in Sprague Dawley rats was performed prior to the *in vivo* micronucleus assay in rats, to assess the MTD of Eleview®. Eleview® was administered as a single IP injection at the doses of 10, 15 and 20 mL/kg. These volumes were selected based on the maximum tolerated volume injectable according to the good practice guide for administration volumes.[20]

Male and female Sprague Dawley rats (two animals/sex/group) were allowed a five-day observation period and were examined regularly for adverse clinical signs until the time of sacrifice (120 h after dosing).

In vivo micronucleus assay in rat bone marrow erythrocytes

The study was in accordance with the current international guidelines.[12,14,21] Six female Sprague Dawley rats/group were treated with a single IP administration of Eleview® at the volumes of 5, 10 or 20 mL/kg or with sterile water at 20 mL/kg. The positive control group received CPA at an oral dose of 20 mg/kg (10 mL/kg).

Rat bone marrow smears were obtained 24 h after administration. Two additional groups of six female rats each were treated with the vehicle at 20 mL/kg or with Eleview® at 20 mL/kg. From these groups, bone marrow smears for evaluation were obtained 48 hours after administration. The sex of the animals and the doses for the micronucleus test were chosen based on the results of the preliminary MTD study. For each animal, the smears were examined for the presence of micronuclei in 4000 polychromatic erythrocytes. The ratio of polychromatic to normochromatic erythrocytes was assessed by examination of at least 500 erythrocytes per animal.

Statistical analysis

The statistical methods used were the Pristima package, including Bartlett's test for homogeneity of variance, Dunnett's test for homogeneous data, and Cochran and Cox's modified t-Test for nonhomogeneous data.

Also, analysis of variance (ANOVA) followed by Dunnett's test, Fischer´s exact test, the χ^2 test were applied and for a single treated group, the Mann-Whitney test was performed. Statistical significance was assessed at a 95% confidence level ($P < 0.05$).

RESULTS

Acute systemic toxicity in the rat

No mortality was observed, and the clinical observations were reported and no clinical signs were seen in all treated animals. Body weights and body weight gains of animals treated with Eleview® IP or orally showed the same trend as controls as described in Figure 1A and 1B. Finally, the post-mortem evaluation did not show gross lesions in any animals.

Subacute systemic toxicity (28 days) in dogs

During the study no deaths were registered, no signs of systemic toxicity, no relevant changes in body weight or food consumption were detected. In dogs no toxicologically meaningful changes in body weight were seen (Figure 2A and 2B).

No signs of systemic toxicity were seen during the study. Sporadically, on day 3 to 5 of study, soft feces were seen in both Eleview® and control treated animals as a possible consequence of the liquid food and laxative given to animals before the endoscopic procedures. None of these findings were seen after Day 5 of study

Table 1: Ames assay in different bacterial strains with metabolic activation[a]

	TA100		TA1535		TA1537		TA98		WP2 uvrA	
Dose μL/plate	**RF**	**Exp I**	**RF**	**Exp I**	**RF**	**Exp I**	**RF**	**Exp I**	**RF**	**Exp I**
5	1.0	1.2	1.2	1.0	0.3*	1.0	1.5	1.1	1.1	0.9
4	1.3	NT	1.4	NT	0.4*	0.9	1.5	1.1	1.0	NT
3	1.1	NT	0.8	NT	0.4*	0.7	1.0	1.0	1.2	NT
2	0.9	NT	1.1	NT	1.1	1.0	1.0	1.1	0.9	NT
1	1.0	NT	1.0	NT	0.4*	0.9	1.1	1.2	1.1	NT
Positive control 2-amino-anthracene	11.6	13.0	11.9	12.5	14.2	11.3	69.4	79.1	8.5	5.1

[a]Fold increase number of revertants relative to vehicle (ratio of treated:vehicle); RF: Range finding assay; Exp I: 1[st]Experiment; NT: Not tested; *Reduced colony numbers

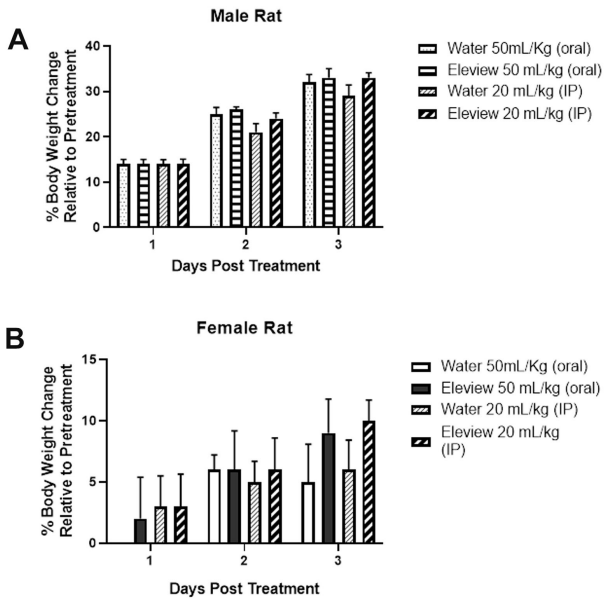

Figure 1. Body weight gain in single dose toxicity study. Body weight-post treatment with Eleview (oral and intraperitoneal) in male (A) and female (B) rats. Histogram reports the mean % of Body Weight change relative to pre-treatment and ± SEM results.

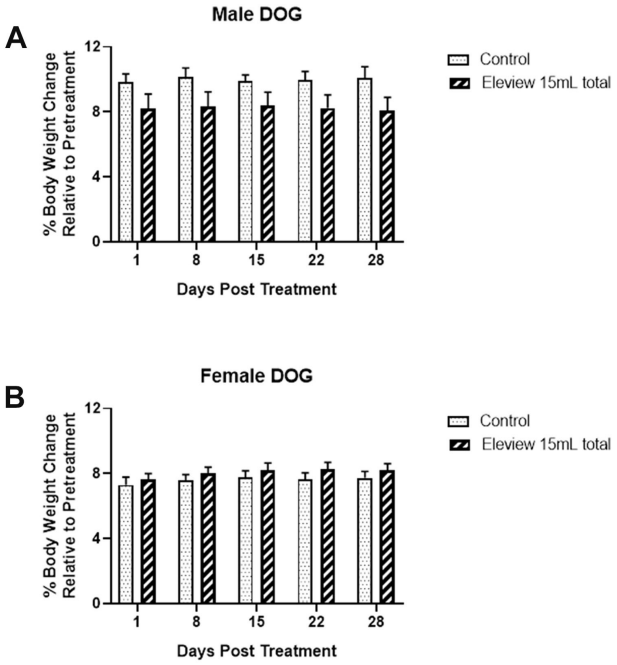

Figure 2. Body Weights post treatment in male dogs. Body weight-post treatment with Eleview 5mL (each injection) per 3 submucosal injection sites (Esophagus, Stomach and Colon wall) in male (A) and female (B) dogs. Histogram reports the mean % body weight value after treatment and ± SEM results. Control: Sterile Water for injection 5 mL (each injection) per 3 submucosal injection sites (Esophagus, Stomach and Colon wall).

both in control and Eleview® treated animals.

Minimal decreases in neutrophils, lymphocytes and monocytes (10%-20%) were observed in both sexes treated with Eleview®. A slight increase in gamma glutamyl transferase was recorded in almost all treated and control animals.

The blood coagulation did not change in treated and control animals. Presence of urine leukocytes and erythrocytes were recorded in most treated and control males and females, respectively, but also during the pre-test occasion. Most of the females, both treated and control, showed a slight decrease in urinary volume. Although present, all the minor changes described are not considered treatment-related but rather reflect normal fluctuations since the values fall within the normal reference range for the species.

At the post-mortem examination, no statistically significant changes were recorded in absolute organ weights at the end of the treatment period in treated animals compared with control groups. No gross lesions, attributed to treatment with the test item, were observed in any animals. Specifically, no macroscopic changes were seen at the injection sites in esophagus, stomach and colon for vehicle or Eleview® treated animals.

There were no microscopic changes that could be

attributed to Eleview® administration. In the esophagus of females very minimal focal periductal inflammatory cell infiltration in both controls and Eleview® treated animals was noted. This change was considered procedure-related and not a reaction to Eleview®. All other histological changes observed in animals treated with Eleview® were considered related to spontaneous background alterations or noted in control animals with a similar incidence and/or severity.

Bacterial reverse mutation study (Ames assay)

No toxicity, in the form of a reduced number of colonies and/or a reduced background lawn, was seen both in the presence and absence of metabolic activation (Table 1 and Table 2).

The results of the bacterial reverse mutation test with Eleview® demonstrated no concentration-related and reproducible increases in revertant colony numbers at any concentration tested in *S. typhimurium* and in *E. coli* WP2 uvrA. All negative controls (vehicle) counts fell within the acceptable range as established for the testing laboratory (current historical mean ± two standard deviations). All positive control chemicals induced large increases in revertant numbers in the appropriate strains, far exceeding the normal historical range.

In vitro chromosome aberration assay

Eleview® did not induce precipitation or cytotoxicity (determined as decrease below 70% relative mitotic index) at any concentrations evaluated. In addition, no aberration rate increases were observed after treatment with Eleview® (Table 3 and Table 4).

In experiment I Eleview® aberration rates were within the historical control data of the testing facility at all the tested concentrations (1.0, 1.5 and 2 μL/mL). In experiment II without metabolic activation, Eleview® aberration rates were within the historical control data of the testing facility at 1.0 and 2 μL/mL. For the concentration of 1.5 μL/mL, Eleview®'s aberration rate of 3.3% was slightly above the upper historical control limit. However, since the increase was not statistically significant, and no dose-response relationship was observed, the effect was considered as not biologically relevant.

No substantial increase in the frequencies of polyploid metaphases was found after treatment with Eleview® compared to the control's frequencies.

No statistically significant increase ($P < 0.05$) of cells with chromosomal aberrations was noted. The χ^2 test for trend was performed to test whether there is a concentration-related increase in chromosomal aberrations. No statistically significant increase was observed in experiment I without and with metabolic activation and in experiment II without metabolic activation.

In all experiments, negative controls provided aberration rates within the historical control data. As expected, positive controls (EMS and CPA) induced biologically and statistically relevant increases of chromosomal aberrations, thus proving the ability of the test system to indicate potential clastogenic effects.

Maximum tolerated dose determination

No mortality, nor significant clinical signs, were seen in all treated animals; only a minimal decrease in the body weight gain was observed at the highest dose both in male and female animals. Based on these results, the administration volume of 20 mL/kg was considered as the MTD.

In vivo micronucleus assay in rats

During the experimental phase, there were no abnormal changes in the animal's general appearance; no significant body weight changes were observed in rats at all tested doses 24 or 48 hours after treatment. No reduction in the ratio of polychromatic to normochromic erythrocytes was observed in comparison to the vehicle control group, at both 24 and 48 hours after treatment; moreover, no statistically significant increase in the frequency of micronucleated polychromatic erythrocytes, compared to vehicle controls, was seen at 24 and 48 hours after treatment (data not shown).

Animals treated with CPA (positive control) showed a statistically significant increase in the number of micronucleated polychromatic erythrocytes compared with vehicle controls (Figure 3). The percentage of cells with micronuclei in the negative control (vehicle) group was within the historical range at the testing laboratory.

DISCUSSION

Submucosal injection is important in the EMR procedure providing a sufficiently high submucosal elevation for safe resection during the ESD procedure.[2] To increase efficacy and safety, EMR and ESD techniques require the injection of an agent underneath the mucosa into the submucosal layer.[22] Submucosal injection solutions separate the lesion from the muscularis propria to allow the complete resection of the lesion and to prevent perforation and thermal injury to the GI wall. Saline-assisted endoscopic mucosal resection is an established therapeutic method and is commonly used in clinical practice because of its low cost and ease of use. However, it is sometimes difficult to maintain a desired level of tissue elevation after

Table 2: Ames assay in different bacterial strains without metabolic activation[a]

	TA100		TA1535		TA1537		TA98		WP2 uvrA	
Dose μL/plate	**RF**	**Exp I**	**RF**	**Exp I**	**RF**	**Exp I**	**RF**	**Exp I**	**RF**	**Exp I**
5	1.1	1.5	0.9	1.6	1.3	1.3	1.9	1.4	0.9	1.2
4	1.1	NT	0.7	NT	0.9	1.0	1.6	1.5	0.9	NT
3	1.0	NT	0.6*	NT	0.9	1.1	1.3	1.1	1.0	NT
2	1.0	NT	0.8	NT	0.7	1.2	1.4	1.4	1.0	NT
1	1.0	NT	0.7	NT	0.7	1.0	1.0	1.1	1.0	NT
Positive control	2.4	2.1	22.2	22.1	39.0	56.5	9.0	4.9	5.0	5.7
Positive control chemical name	Sodium azide				9-aminoacridine		2-nitrofluorene		Methyl methane-sulfonate	

[a]Fold increase number of revertants relative to vehicle (ratio of treated:vehicle); RF: Range finding assay; Exp I: 1[st]Experiment; NT: Not tested; *Reduced colony numbers

Table 3: *In vitro* chromosome aberration assay without and with metabolic activation 4 h treatment, 21 h preparation interval experiment I

Condition	Eleview concentration (μL/mL)	Relative increase cell count[a] (%)	Mean % aberrant cells including gaps	Mean % aberrant cells excluding gaps	Historical laboratory negative control range	Statistical significance relative to negative control[b]
Without metabolic activation	0	100	4.3	2.0	From -0.32% to 3.54%	NA
	1.0	89	2.0	0.3		-
	1.5	107	3.3	2.7		-
	2	75	2.3	1.0		-
	EMS pos. cont.	74	8.3	5.7		+
With metabolic activation	0	100	6.3	3.0	From 0.02% to 3.76%	NA
	1.0	86	4.7	2.3		-
	1.5	100	1.0	2.3		-
	2.0	102	5.3	2.0		-
	CPA pos. cont.	87	17.7	16.9		+

EMS: ethylmethane sulfonate; CPA: cyclophosphamide; [a]Relative increase in cell count calculated by the increase in cell number of the test group compared to the negative control group; NA: not applicable; pos con: positive control; [b]Statistically significant increase compared to negative controls (Fisher's exact test, $P < 0.05$), +: significant; -: not significant

Table 4: *In vitro* chromosome aberration assay results, experiment II

Condition	Eleview concentration (μL/mL)	Relative increase cell count[a] (%)	Mean % aberrant cells including gaps	Mean % aberrant cells excluding gaps	Historical laboratory negative control range	Statistical significance relative to negative control[b]
Without metabolic activation	0	100	3.3	2	From -0.47% to 3.01%	NA
	1.0	134	4	2.7		-
	1.5	128	5	3.3		-
	2	111	3.7	2.3		-
	EMS pos. cont.	88	32	30		+

[a]Relative increase in cell count calculated by the increase in cell number of the test group compared to the negative control group; [b]Statistically significant increase compared to negative controls (Fisher's exact test, $P < 0.05$); EMS: ethylmethane sulfonate; NA: not applicable; pos con: positive control; +: significant; -: not significant

injection of NS and its use is hampered by the quick absorption of the solution into the surrounding tissue, thereby resulting in the need for repeated injections.

In recent years, various submucosal injection solutions have been developed and studied for safety and efficacy. Eleview®, is a medical device which provides a tool to endoscopists to perform fast and safe excision of

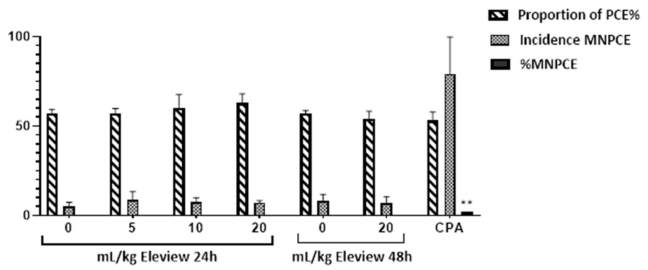

Figure 3. *In vivo* micronucleus assay in rats. Histogram reports the value of parameters of micronucleus assay for Eleview after 24 h and 48 h and cyclophosphamide 24 h as positive control. 0: vehicle, 5, 10, 20 mL/kg Eleview; CPA: cyclophosphamide 20 mg/kg; PCE: polychromatic erythrocytes; MPCE: number of micronucleate polychromatic erythrocytes observed per 4000 polychromatic erythrocytes examined; **Statistically Significant relative to vehicle ($P < 0.05$), Mann-Whitney test.

adenomas or polyps during endoscopy. It is injected between the colonic mucosal layers where it clearly separates the mucosal layers for more than 60 minutes. The composition is stained with methylene blue thus allowing a clear tissue differentiation. These characteristics allow sufficient time for resection and greatly reduces the risk of colon perforation during the procedure.

To comply with the national and international guidelines and regulations of medical devices, including ISO 10993 guidelines, a meticulous and detailed efficacy and safety assessment of Eleview® was performed *in vitro* and in animal models.

As published by Spadaccini *et al.* [3] in an *in vivo* porcine model, Eleview® resulted in higher efficacy when compared to saline solution containing methylene blue at 0.001%; still considered as the standard of care for endoscopy procedures. Furthermore, they compared Eleview® with saline solution and the microemulsion appeared to be safer for intra-procedural adverse events, and at least as safe for long-term safety outcomes, such as post-resection site healing and intramural inflammation. Notably, no adverse events were observed in the same study.

Most importantly, a double-blind randomized clinical study was conducted to compare Eleview® to saline solution in the performance of EMR procedures of colorectal polyps larger than 2 cm; the study demonstrated that Eleview® was more effective than and was as safe and as easy to use as saline solution.[4]

In recent years, concerns were raised over a potential genotoxic effect of methylene blue in chromoendoscopy procedures.[8,9] Because Eleview® contains methylene blue in trace amounts an extensive genotoxicity assessment program was performed to confirm the safety of the product. The program comprised acute systemic toxicity in rats and subacute toxicity tests in Beagle dogs, Ames assay, chromosome aberration assay and *in vivo* micronucleus assay in rats. Eleview®, ready to use commercially available emulsion, was administered orally or IP at the maximum applicable volumes of 50 mg/kg or 20 mg/kg, respectively, to Sprague Dawley rats of both sexes and did not induce any systemic acute toxicity.

When administered as a single submucosal injection in the esophagus, stomach and colon in a volume of 5 mL/site, for a total of 15 mL/animal, to Beagle dogs of both sexes, Eleview® did not cause any relevant in-life observations. Also, Eleview® did not cause histopathological changes in any organs/tissues or in the injection site.

A preliminary MTD study was performed in rats to ensure the maximal exposure to the bone marrow as the target in the *in vivo* micronucleus test. These studies were performed in accordance with internationally recognized and accepted standards and were performed under GLPs rules (except for the MTD study). Eleview® tested in the Ames assay, demonstrated no concentration-related increases in revertant colony numbers. In the chromosomal aberration test, no precipitation nor cytotoxic effects were observed. Eleview® was non-genotoxic in the micronucleus test and no clastogenic effects were observed. Collectively, these results establish that Eleview® does not induce any systemic acute toxicity or subacute systemic toxicity and it is not genotoxic or mutagenic. Coupled with its superior efficacy over standard saline solution for endoscopic resection of colorectal lesions, Eleview® is a safe and effective medical device to use in clinical practice.

DECLARATIONS

Author contributions

Luigi Longo conducted the analyses, drafted sections of the manuscript and edited the manuscript. Mara Gerloni conducted the analyses and edited the manuscript. Gordon Alton drafted sections of the manuscript, edited and approved the final and submitted version. Alberto Morisetti conducted the analyses, drafted sections of the manuscript and edited the manuscript.

Conflicts of interest

There is no conflict of interest among the authors.

Data sharing statement

No additional data is available.

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