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Optimising Mitomycin C Activity During Intravesical Instillation

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Summary. Walker 256 carcinosarcoma was shown to be sensitive to mitomycin C in vitro. In order to make practical recommendations for the clinical intravesical use of mitomycin C, this cell line was used to evaluate the activity of mitomycin C under different conditions. Mitomycin C loses its antitumour activity rapidly at pH's below 6. At higher pH's (up to 10) mitomycin C is stable and suitable for intravesical application. To secure a sufficiently high pH in the bladder during intravesical treatment, phosphate buffer 0.05 M with a pH of 7.4 is recommended. Constituents of urine very little decrease the activity of buffered mitomycin C during a 2 h application. Prednisolone, which has been suggested to prevent the harmful chemical cystitis, has no inhibitory effect on the activity of mitomycin C.

Key words: Mitomycin C, Instillation therapy, Superficial bladder cancer, Antitumour activity, Urinary pH

Introduction

Mitomycin C is an aminoquinone antibiotic isolated from Streptomyces caespitosus in 1956. It is effective against many human malignancies [1]. For 15 years mitomycin C has been used as local instillation therapy for superficial bladder cancers [7]. In 1977 Kato showed that in a solution containing 0.02–1 μg of mitomycin C per ml half of the T-24 cells (line isolated from human bladder transitional cell carcinoma) died in 2 h [7].

Adenosine triphosphate is the basic energy source of living cells and not found in dead cells [4]. In our study we have measured the ATP content of Walker 256 carcinosarcoma cells in experimental conditions after mitomycin C incubations to evaluate the sensitivity of the cell line in these conditions.

The pH of human urine may vary between 4.6 and 8.2 [9]. It is presumed that the extreme pH's may affect the stability of the cytostatic drug during instillation.

The purpose of the present study was to determine the proper instillation conditions by measuring the effects of urinary pH and prednisolone on the antitumour activity of mitomycin C and to make practical recommendations for the use of intravesical mitomycin C in the management of superficial bladder tumours.

Materials and Methods

Luminometer 1250 (LKB-Wallac, Turku, Finland) equipped with LKB 2210 potentiometric recorder was used for the determination of ATP.

ATP monitoring reagent^R (LKB-Wallac, Turku, Finland), containing purified firefly luciferase and Tris-HCl buffer (0.25 M, pH 7.75) were needed for light reaction in bioluminescence assay. TCA (1%) was used to release the intracellular ATP from the cells. Mitomycin C was obtained from Bristol-Myers Company, and prednisolone from Sigma Chemical Company.

The following buffers (reagents obtained from Baker or Merck) were used: citrate-HCl (pH 2.0, 4.0, 5.0 and 6.0), $Mg(OH)_2$-$NaHCO_3$ (pH 6.0 and 7.4), citric acid-phosphate (pH 6.0 and 7.0), phosphate buffer (KH_2PO_4-Na_2HPO_4) (pH 7.0, 7.4 and 8.0), glycine-NaOH (pH 10.0) and phosphate – buffered saline (pH 7.4). The buffers were prepared according to Stauff and Jaenicke [10], except $Mg(OH)_2$-$NaHCO_3$ which was titrated to a given pH by 0.5 M $NaHCO_3$-solution after solubilization of $Mg(OH)_2$ in HCl.

The cell line used in this study was rat Walker 256 carcinosarcoma. A solid tumour was removed from the subcutaneous space, cut into small pieces with a scalpel on a petri dish and spread out in the medium. The cell line was routinely maintained on RPMI 1640 (with 10% foetal calf serum L-glutamine 292 mg/l, and penicillin and streptomycin 100 IU/ml and 100 μg/ml, respectively).

Determination of Mitomycin C Activity

The sensitivity of Walker carcinosarcoma 256 to mitomycin C was studied in vitro by incubating cells in the presence of different concentrations of mitomycin C (0, 0.01, 0.1, 1.0 and 5.0 μg/ml) in microtitre plates in the incubator (5% CO_2 + 95% air, 37 °C) for 24 and 48 h. Cell viability was determined at each time by bioluminescence, i.e. by measuring adenosinetriphosphate, the basic energy source of the cells. The method has been described in detail in a

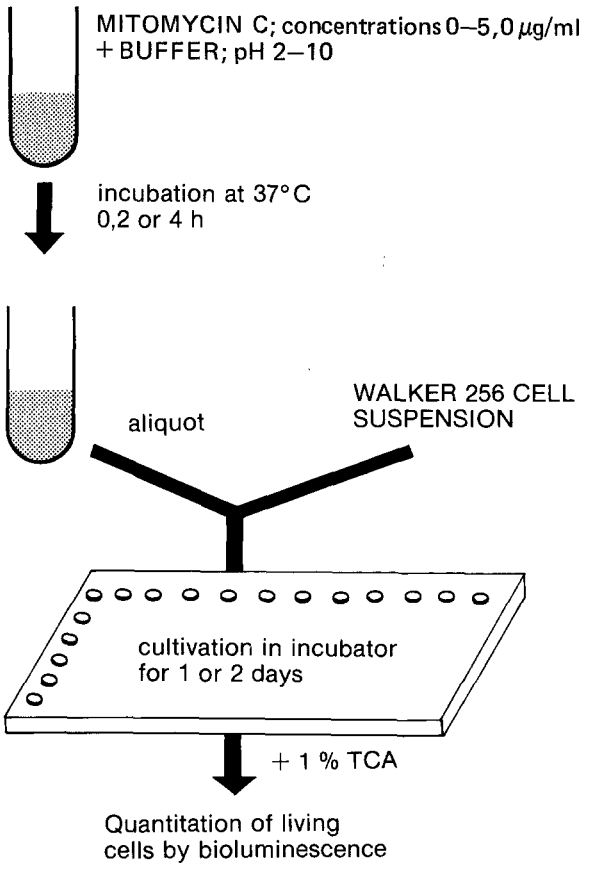

Fig. 1. Experimental design for testing mitomycin C stability

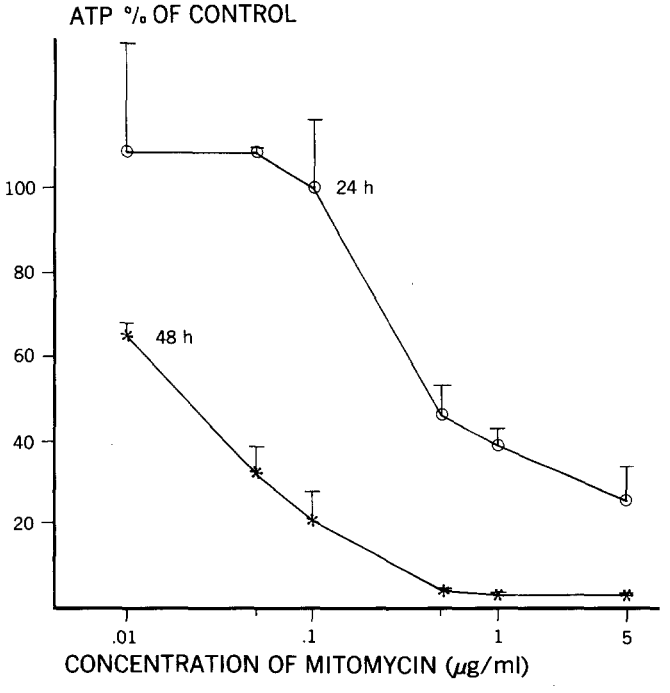

Fig. 2. Cytostatic effect of mitomycin C on Walker 256 carcinosarcoma measured by bioluminescence after 1 and 2 days growth in vitro. Each point is mean of three independent measurements

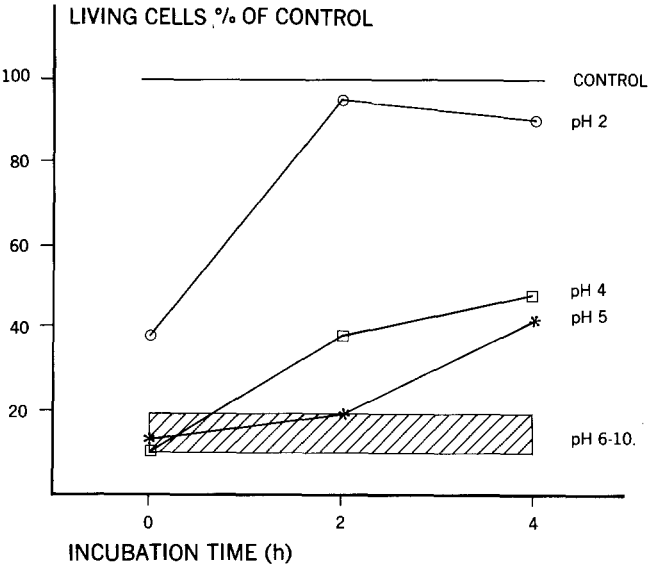

Fig. 3. Effect of pH on the cytostatic activity of mitomycin C (means of two independent measurements). Amount of living cells was measured by bioluminescence: Mitomycin C was incubated 0, 2 or 4 h in buffers at the given pH's. Control: no mitomycin C. On the shaded area are situated all results with buffers pH 6, 7, 7.4, 8 and 10

forthcoming paper [4] and is shortly as follows: cell suspension on microtitre plates is extracted for ATP by 1% TCA (100 µl suspension + 100 µl TCA solution) and mixed well with Finnpipette. 100 µl of the mixture is pipetted into measuring cuvettes containing buffered ATP monitoring reagent^R (400 µl). Bioluminescence is read directly after a short mixing by luminometer.

Testing of Mitomycin C Stability at Different pH's

The experimental procedure for testing mitomycin C stability is shown in Fig. 1.

Results

The sensitivity of Walker 256 carcinosarcoma to mitomycin C is illustrated in Fig. 2. It is evident that mitomycin C decreases cell viability in a dose- and time-dependent manner.

Cytostatic activity of mitomycin C after incubation for 0, 2 and 4 h in buffers with different pH (2–10) is presented in Fig. 3. At pH equal to or higher than 6, mitomycin C is stable at least for 4 h. In acidic conditions mitomycin C rapidly loses its cell-killing activity.

Search for a clinically useful buffer is presented in Table 1 and Fig. 4. The ideal buffer does not inactivate mitomycin C, may be instilled without bladder irritation, and has adequate buffering capacity to keep a sufficiently alkaline pH in the bladder during treatment. Table 1 shows that many buffers might be clinically useful (best perhaps $Mg(OH)_2$-$NaHCO_3$ buffer). It also shows that the activity of mitomycin C is not diminished in the presence of urine (10%). The final selection of the buffer results from Fig. 4: if titrated with human urine (pH 5.26), phosphate buffers can most effectively withstand the decrease of pH and are therefore recommended.

Table 1. Decrease of cytostatic activity of mitomycin C during incubation (0, 2 and 4h) in different buffers at pH 5–10. The drug activity was measured by bioluminescence using Walker 256 carcinosarcoma cells in vitro, and is expressed as percentages of zero-incubations. Different concentrations of mitomycin C (0.1 and 0.5 μg/ml) and different growth times (24 and 48 h) have been combined (n = 12 at each point if not stated otherwise)

Incubation buffer		Activity of mitomycin C (% of zero-incubation) (mean ± sd)		
		0 h	2 h	4 h
Phosphate	pH 6	100	92 ± 3	85 ± 14
	pH 7	100	91 ± 9	96 ± 6
	+ 10% urine	100	94 ± 8 (n = 6)	89 ± 3 (n = 6)
	pH 8	100	91 ± 8	90 ± 9
Citrate-phosphate	pH 5	100	80 ± 1	52 ± 25
	pH 6	100	90 ± 11	89 ± 10
	pH 7	100	94 ± 11	90 ± 13
	+ 10% urine	100	93 ± 9 (n = 6)	91 ± 11 (n = 6)
$Mg(OH)_2$-$NaHCO_3$	pH 7	100	113 ± 12	100 ± 22
	pH 8	100	100 ± 0 (n = 4)	95 ± 19 (n = 4)
PBS	pH 7.4	100	100 ± 0 (n = 4)	95 ± 8 (n = 4)
	+ 10% urine	100	97 ± 6	94 ± 8
Saline		100	91 ± 14	93 ± 8
Glycine-NaOH	pH 10	100	100 ± 4 (n = 4)	95 ± 6 (n = 4)

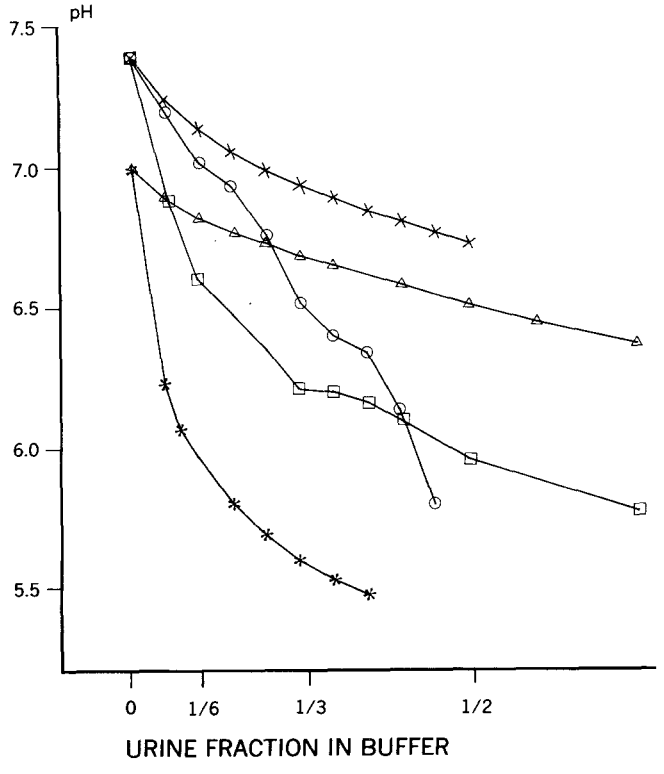

Fig. 4. Effect of human urine, pH 5.26, collected at daytime, on the pH of the investigated buffers. 10 ml buffer (0.05 M) was titrated with the urine. The phosphate buffers are expected to retain sufficiently high pH in the bladder at all conditions during intravesical instillation x = Phosphate 7.4; ○ = PBS 7.4; □ =$Mg(OH)_2$-$NaHCO_3$ 7.4; △ = phosphate 7.0; * = $Mg(OH)_2$-$NaHCO_3$ 7.0

Prednisolone slightly inhibited cell growth at high concentrations but had no influence on the activity of mitomycin C, as shown in Table 2.

Discussion

Mitomycin C has three potentially active groups: quinone, urethane and aziridine ring [6]. It has been shown that mitomycin C has to be first activated and that all the tissues studied so far seem able to do that [1], including normal urinary bladder epithelium [2]. In our study (Fig. 2) mitomycin C is already activated in the cell culture, and thus the activating factor is at a cellular level.

In the manufacturer's instructions Ogawa and Mishina recommended $Mg(OH)_2$-$NaHCO_3$ buffer in connection with intravesical mitomycin C treatment [8]. Eksborg et al. [3] suggested the use of a phosphate buffer in connection with adriamycin treatment in order to retain a suitable pH. In our study mitomycin C rapidly lost antitumour activity at pH's below 6. At higher pH's (up to 10) mitomycin C was stable, active and suitable for intravesical instillation therapy (Fig. 3). The 0.05 M phosphate buffer with pH 7.4 would seem suitable for clinical use of mitomycin C (Fig. 4).

In controlled conditions, addition of urine (pH 5.26, 10%) does not significantly affect the antitumour activity of buffered mitomycin C (Table 1).

Its a well known fact that instillation therapy is sometimes limited by a chemical cystitis. Prednisolone may prevent this side effect [5]. In our study prednisolone did

Table 2. Effect of combination of prednisolone (P) and mitomycin C (MC) on Walker 256 carcinosarcoma cells in vitro. Prednisolone slightly inhibits cell growth at high concentrations and has no influence on the effect of mitomycin C. Cells were cultivated for 2 days

Intracellular ATP level (mV) (both duplicates given)

	Concentration of					
	P (mol/l)		P + MC 0.1 μg/ml		P + MC 0.5 μg/ml	
No	13.1	13.4	1.0	1.3	0.4	0.4
10^{-8}	13.4	13.2	1.3	1.2	0.4	0.4
10^{-7}	12.1	12.5	1.2	1.3	0.35	0.35
10^{-6}	10.0	10.8	1.3	1.5	0.4	0.4
10^{-5}	11.3	10.7	0.9	0.8	0.4	0.4
10^{-4}	7.8	7.4	1.3	1.1	0.4	0.4

not diminish in vitro the antitumour effect of mitomycin C (Table 2) and can therefore be used together with mitomycin C in instillation therapy.

Conclusion

Mitomycin C loses its antitumour activity at pH's below 6. Phosphate buffers can withstand most effectively the decrease of pH and are therefore recommended for use with mitomycin C. Prednisolone and urine do not diminish the local antitumour effect of mitomycin C.

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