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2 **Development of a new application of the comet assay to assess levels of**  
3 **O<sup>6</sup>-methylguanine in genomic DNA (CoMeth)**

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17

18 **Abstract**

19 O<sup>6</sup>meG is one of the most pre-mutagenic, pre-carcinogenic and pre-cytotoxic DNA  
20 lesion formed by alkylating agents. Repair of this DNA damage is achieved by the  
21 protein MGMT, which transfers the alkyl groups from the O<sup>6</sup> position of guanine to a  
22 cysteine residue in the active centre of MGMT. Since O<sup>6</sup>meG repair by MGMT, is a  
23 stoichiometric reaction that irreversibly inactivates MGMT, which is subsequently  
24 degraded, the repair capacity of O<sup>6</sup>meG lesions is dependent on existing MGMT active  
25 molecules. In the absence of active MGMT, O<sup>6</sup>meG is not repaired and during  
26 replication, O<sup>6</sup>meG:T mispairs are formed. MMR system recognizes these mispairs and  
27 introduces a gap into the strand. If O<sup>6</sup>meG remains in one of the template strands the  
28 futile MMR repair process will be repeated, generating more SB. The toxicity of O<sup>6</sup>meG  
29 is, therefore, dependent on MMR and DNA SB induction of cell death. MGMT, on the  
30 other hand, protects against O<sup>6</sup>meG toxicity by removing the methyl residue from the  
31 guanine. While removal of O<sup>6</sup>meG makes MGMT an important anticarcinogenic  
32 mechanism of DNA repair its activity significantly decreases the efficacy of cancer  
33 chemotherapeutic drugs that aim at achieving cell death through the action of the MMR  
34 system on unrepaired O<sup>6</sup>meG lesions.

35 Here, we report on a modification of the comet assay (CoMeth) that allows the  
36 qualitative assessment of O<sup>6</sup>meG lesions after their conversion to strand breaks in  
37 proliferating MMR proficient cells after MGMT inhibition. This functional assay allows  
38 the testing of compounds with effects on O<sup>6</sup>meG levels, as well as on MGMT or MMR  
39 activity in a proliferating cell system. The expression of MGMT and MMR genes is  
40 often altered by promoter methylation and new epigenetically active compounds are  
41 being designed to increase chemotherapeutic efficacy. The CoMeth assay allows the  
42 testing of compounds with effects on O<sup>6</sup>meG, MGMT or MMR activity. This  
43 proliferating cells system complements other methodologies that look at effects on these  
44 parameters individually through analytical chemistry or in vitro assays with  
45 recombinant proteins.

46 **Keywords:** comet assay, O<sup>6</sup>meG lesion, MGMT, MMR system, alkylating  
47 chemotherapy

48 **Abbreviations:** Base excision repair (BER); O<sup>6</sup>-benzylguanine (BG); Methoxyamine  
49 (Mx); Methyl methanesulfonate (MMS); Mismatch repair (MMR); N-methyl-N-  
50 nitrosourea (MNU);

51 N-methylpurine-DNA glycosylase (MPG); O<sup>6</sup>-methylguanine (O<sup>6</sup>meG); O<sup>6</sup>-  
52 methylguanine-methyltransferase (MGMT); Strand breaks (SB).

53

54

## 55 **1. Introduction**

56 Alkylating agents are ubiquitous. They are present in the environment (e.g. cigarette  
57 smoke and fuel combustion), diet (e.g. presence of nitrosamines in food), or are  
58 endogenously produced [1, 2]. Although generally in low concentrations, alkylating  
59 agents may be mutagenic and carcinogenic [3]. Another source of human exposure to  
60 alkylating agents is cancer chemotherapy [4] where several alkylating agents are used  
61 due to their ability to induce extensive DNA damage and cell death.

62 Alkylating agents can react with different nucleophilic atoms on the DNA bases,  
63 inducing a large amount of DNA lesions. Of the various types of alkylation damage, N-  
64 alkylated adducts, such as N<sup>7</sup>-methylguanine (N<sup>7</sup>meG), N<sup>3</sup>-methyladenine (N<sup>3</sup>meA) and  
65 N<sup>3</sup>-methylguanine (N<sup>3</sup>meG) are the most abundant (more than 80% of alkylated bases).  
66 O-alkylated adducts are less abundant (less than 10% of the total alkylated bases) and  
67 include O<sup>6</sup>-methylguanine (O<sup>6</sup>meG) and O<sup>4</sup>-methylthymine (O<sup>4</sup>meT) [5, 6]. In general,  
68 O-alkylations are highly mutagenic and genotoxic, whereas N-alkylations are cytotoxic,  
69 but less mutagenic[1].

70 The biological effect of these damages depends on the balance between the DNA repair  
71 ability of the cell and the extent of the damage [7]. The most important DNA repair  
72 systems involved in the repair of alkylating damage are: the O<sup>6</sup>-alkylguanine-DNA-  
73 alkyltransferase (MGMT), the DNA mismatch repair (MMR) system, and the base  
74 excision repair (BER) system. The repair of O<sup>6</sup>meG is achieved by the protein MGMT,  
75 which transfers the alkyl groups from the O<sup>6</sup> position of guanine, and to a lesser extent  
76 from the O<sup>4</sup> position of thymine, to a cysteine residue in the active centre of the MGMT  
77 molecule. This reaction irreversibly inactivates MGMT that is ubiquitinated and  
78 degraded in the proteasome [8]. Since O<sup>6</sup>meG repair by MGMT is a stoichiometric  
79 reaction, the repair capacity of O<sup>6</sup>meG is dependent on the number of existing active  
80 MGMT molecules [4, 9]. In the absence of active MGMT, O<sup>6</sup>meG is not repaired by the  
81 cell and during replication pairs with thymine instead of cytosine resulting in O<sup>6</sup>meG:T  
82 mispairs. In mismatch repair (MMR) proficient cells, the O<sup>6</sup>meG:T mispair is  
83 recognized by the MMR proteins and the new thymine is removed introducing a gap  
84 into the strand. In the next round of replication another thymine is mispaired with  
85 O<sup>6</sup>meG that will again be removed by MMR. Recognition by MMR creates a gap in  
86 DNA by incision in the new replicated strand. If O<sup>6</sup>meG remains in one of the template  
87 strands the MMR repair process will be repeated, creating a “futile repair loop”. This  
88 loop will eventually result in toxic double-strand breaks leading to chromosomal

89 aberrations, cell-cycle arrest or apoptosis [4, 6, 10-12]. Failure to repair O<sup>6</sup>meG causes  
90 GC-AT transitions [13]. This lesion results in point mutations that may initiate the  
91 carcinogenic process (Fig. 1) [7, 14].

92 MGMT and MMR have contrasting effects on DNA O<sup>6</sup>meG. While MGMT is an  
93 efficient mechanism of repair, MMR in contrast, does not remove the methylated base  
94 but introduces more lesions, such as strand breaks (SB), in a futile attempt to repair the  
95 mismatch that results in induction of cell death. In MMR deficient cells O<sup>6</sup>meG fails to  
96 cause apoptosis. Resistance to cell death induction by alkylating agents can be mediated  
97 by both MGMT and MMR. Active MGMT and loss of the MMR pathway protect  
98 cancer cells against the cell death induced by methylating chemotherapeutic drugs,  
99 while increasing the drug's mutagenicity [15]. Depletion of MGMT activity (for  
100 example, by O<sup>6</sup>-benzylguanine (BG) or by epigenetic silencing of the MGMT gene)  
101 with an intact MMR system results, on the other hand, in reversion of resistance with  
102 high sensitivity to the cytotoxic effects of alkylating drugs [12, 16, 17]. MGMT  
103 inhibitors are frequently used in combination with alkylating drugs to increase  
104 therapeutic efficacy of alkylating agents in tumors that express MGMT [18]. In contrast  
105 with responsiveness to chemotherapeutic agents, an increase in MGMT produced for  
106 instance by dietary constituents may have a beneficial cancer preventive effect [19].

107 N-methyl-N-nitrosourea (MNU) is an SN1-type alkylating agent that induces O<sup>6</sup>meG  
108 lesions. Repair of these lesions by MGMT provides protection against MNU toxicity  
109 [20]. Depletion of MGMT by BG, a specific inhibitor that acts as a pseudosubstrate,  
110 potentiates the toxicity of this alkylating agent in MMR proficient backgrounds  
111 increasing treatment efficacy [21]. Methyl methanesulfonate (MMS) is another  
112 alkylating agent but of the SN2-type that mainly alkylates nitrogens. In this case,  
113 O<sup>6</sup>meG generated corresponds only to around 0.3% of the total alkylating damages [5].

114 The repair reaction of O<sup>6</sup>meG by MGMT leads to stoichiometric (1:1) inactivation of  
115 MGMT. Therefore the number of inactivated MGMT molecules corresponds to the  
116 number of O<sup>6</sup>meG lesions repaired. Currently, O<sup>6</sup>meG levels are measured by [<sup>3</sup>H]-  
117 based O<sup>6</sup>-alkylguanine-DNA alkyltransferase inactivation assay or by HPLC [22, 23].  
118 Christmann and collaborators [24], in a recent review, describe and compare different  
119 methods to detect MGMT: MGMT activity, MGMT promoter methylation and MGMT  
120 protein by immunohistochemistry. Simple, less toxic, and cheaper methods need to be  
121 developed for measurement of O<sup>6</sup>meG levels and evaluation of possible effects of

122 chemotherapeutic drugs and/or chemopreventive agents on MGMT activity that also  
123 allow to screen the effects on silencing of MGMT or MMR by promoter methylation.  
124 Single cell gel electrophoresis (SCGE) assay or the comet assay is a simple, fast and  
125 low cost method to assess DNA damage [25, 26]. The comet assay is widely used to  
126 measure oxidatively damaged DNA where it allows the evaluation of effects of  
127 environmental factors and chemoprevention by dietary constituents [27, 28]. Alkylating  
128 DNA damage occurs more frequently than oxidative damage and is also a more relevant  
129 driver of mutagenesis or inducer of cell death in chemotherapy. Beside oxidant agents,  
130 comet assay has been also used to assess strand breaks induced by alkylating agents [29,  
131 30]. However, its application to estimate the levels of O<sup>6</sup>meG bases has not been  
132 explored. In the present study we modified the comet assay in order to enable it to  
133 assess O<sup>6</sup>meG, after its conversion into SB in MGMT inactivated and MMR proficient  
134 cells. We demonstrate that by this O<sup>6</sup>methylation specific variant of the comet assay –  
135 CoMeth assay- O<sup>6</sup>meG levels can be assessed, in proliferating and MMR proficient  
136 cells.

137

## 138 **2. Materials and methods**

### 139 *2.1. Chemicals*

140 MNU, BG, MMS, methoxyamine (Mx), Dulbecco's Modified Eagle Medium (DMEM),  
141 penicillin/streptomycin and trypsin solution were purchased from Sigma-Aldrich (St.  
142 Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Biochrom KG  
143 (Berlin, Germany). SYBR Gold (nucleic acid gel stain) was from Invitrogen Molecular  
144 probes (Oregon, USA). Protein quantification kit was purchased from Bio-Rad  
145 Laboratories, Inc., (Hercules, CA). Monoclonal anti-MGMT and anti-actin were  
146 purchased from Sigma-Aldrich. Peroxidase-conjugated goat anti-mouse antibody, and  
147 Immobilon western blotting detection reagents were purchased from Santa Cruz  
148 Biotechnology, Inc. (Santa Cruz, CA) and Millipore (Billerica, MA), respectively. All  
149 other reagents and chemicals used were of analytical grade.

150

### 151 *2.2. Cell culture*

152 Caco-2 cells (derived from human colon carcinoma) and HCT116 cells were maintained  
153 as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented  
154 with 10% FBS and antibiotics (100U/ml penicillin and 100µg/ml streptomycin), under  
155 an atmosphere of 5% CO<sub>2</sub> at 37°C. Cells were trypsinized when nearly confluent.

156 Stock solutions of MNU were prepared in dimethyl sulphoxide (DMSO), while BG and  
157 MMS were prepared in PBS and aliquots kept at -20°C. The final concentration of  
158 DMSO in medium was <0.5%. The controls received DMSO or PBS according to the  
159 compound in study.

160 Cells were seeded onto 6-well plates (western blot) or 12-well plates (TUNEL and  
161 comet assay) with 2 or 1 ml/well, respectively, at a density of  $0.1 \times 10^6$  cells/ml.  
162 Twenty-four hours after plating, the medium was discarded and fresh medium  
163 containing BG (100µM) to inhibit MGMT was added. Two hours later, MNU (500µM)  
164 was added to induce alkylating damages, without changing the medium. Cells were also  
165 treated only with BG, MNU or DMSO as controls. For each assay, cells were collected  
166 after specific times of incubation as described below.

167

### 168 2.3. Apoptosis assay

169 The effect of BG and/or MNU treatment on induction of apoptosis in Caco-2 and  
170 HCT116 cells was assessed by TUNEL assay. The number of apoptotic cells was  
171 counted after 48, 72 and 96h of MNU incubation as previously described [31].

172

### 173 2.4. MGMT protein expression

174 MGMT expression in Caco-2 cells was monitored by western blotting after 24, 48 and  
175 72h of MNU incubation (with or without BG treatment). The effect of different  
176 concentrations of BG on MGMT protein expression was also measured in Caco-2 cells  
177 after 72h of incubation.

178 Protein concentration was measured with the DC protein assay following the  
179 manufacturer's instructions and 20µg/well were separated on 12% SDS-PAGE and  
180 transferred to PVDF membranes. Membranes were blocked and incubated with the  
181 monoclonal anti-MGMT antibody (1:4,000 dilution) overnight, and then incubated with  
182 the secondary antibody for 1 h at room temperature. Immunoreactive bands were acquired  
183 using the Chemidoc camera (BioRad) and band area intensity quantified by Quantity  
184 One software (BioRad). The results were expressed as percentage of control (cells  
185 without any treatment).

186

### 187 2.5. Assessment of $O^6$ meG levels by the comet assay in MMR efficient cells

188 To verify if the comet assay can be applied to assess the O<sup>6</sup>meG levels in single cells  
189 and to estimate MGMT activity, two colon cell lines were used: Caco-2 cells as MMR  
190 efficient cells and HCT116 cells as a MMR deficient cell line.

191 After 24, 48 and 72h of incubation of Caco-2 or HCT116 cells with MNU (with or  
192 without BG) DNA damage was assessed by the comet assay as previously described  
193 [28, 32]. Briefly, after treatment, Caco-2 and HCT116 cells were trypsinized, washed,  
194 centrifuged, and the pellet suspended in low melting point agarose; about 2x10<sup>4</sup> cells  
195 were placed on a slide (pre-coated with 1% normal melting point agarose and dried) and  
196 covered with a coverslip. After 10 min at 4 °C, the coverslips were removed and slides  
197 were placed in lysis solution (2.5 M NaCl, 100 mM Na<sub>2</sub>EDTA, 10 mM Tris Base, pH  
198 10 plus 1% Triton X-100) for 1h at 4°C. Slides were then placed in a horizontal  
199 electrophoresis chamber with electrophoresis solution (300 mM NaOH, 1 mM  
200 Na<sub>2</sub>EDTA, pH >13) for 40 min at 4°C for the DNA to unwind before electrophoresis  
201 was run for 20 min at 0,8V/cm and ~300 mA. After electrophoresis, slides were washed  
202 two times with PBS and dried at room temperature. For analysis of the comet images,  
203 slides were stained with SYBR Gold solution for 30 min at 4°C; after drying, slides  
204 were analyzed in a fluorescence microscope and Comet IV analysis system (Perceptive  
205 Instruments Ltd, Haverhill, UK) was used to calculate the parameter % of DNA in tail.  
206 About 100 randomly selected cells were analyzed per sample.

207

#### 208 2.5.1. *Effect of BG on O<sup>6</sup>meG levels in Caco-2 cells assessed by the comet assay*

209 To assess the effect of BG on O<sup>6</sup>meG levels, Caco-2 cells were treated with different  
210 concentrations of BG (0-100µM), followed by the addition of 500µM MNU two hours  
211 later (controls received vehicle only) without medium change. After 72h of MNU  
212 incubation DNA damage was assessed by the comet assay as above.

213

#### 214 2.5.2. *Assessment of MMS-induced O<sup>6</sup>meG levels by the comet assay in MMR-* 215 *proficient cells*

216 To assess the ability of our system to assess O<sup>6</sup>meG levels by comet assay induced by  
217 different alkylating agents, Caco-2 cells were incubated with different concentrations of  
218 BG before treatment with MMS (200 µM). DNA damage was assessed 1and 72h after  
219 MMS treatment.

220

#### 221 2.6. *Statistical analysis*



222 Results were expressed as mean  $\pm$  SEM from at least 4 independent experiments.  
223 Significant differences ( $P<0.05$ ) were evaluated by ANOVA or Student's t-test, as  
224 appropriate.

225

### 226 **3. Results**

#### 227 *3.1. Assessment of MNU-induced O<sup>6</sup>meG levels by the comet assay in MMR efficient* 228 *cells*

229 We hypothesized that the comet assay can be used to assess the levels of O<sup>6</sup>meG in  
230 DNA induced by alkylating agents, such as MNU, in MMR proficient cells. Unrepaired  
231 O<sup>6</sup>meG damage, due to the inactivation of MGMT in the presence of BG, would be  
232 recognized by the MMR pathway, in MMR-proficient cells, upon cell replication. In  
233 this process, the MMR system introduces a SB at sites of damage that can be recognized  
234 by the comet assay and % DNA in tails will be higher as the extent of MGMT inhibition  
235 increases.

236 To test this hypothesis, Caco-2 cells – MMR-proficient – were treated with the  
237 alkylating agent MNU in the presence or absence of the MGMT inhibitor BG. DNA  
238 damage was assessed by the comet assay at different incubation times with MNU. As  
239 shown in Fig. 2, after one hour of incubation with MNU, significant DNA damage was  
240 detected with and without the presence of BG. DNA damage decreased with increasing  
241 time, which corresponds to the repair of the initial DNA damage (such as SB, AP sites)  
242 induced by MNU. After 72hr, the levels of DNA damage reached control values of cells  
243 without MNU or BG treatment. However, in Caco-2 cells in the presence of BG, DNA  
244 damage detectable by the comet assay was still significant and it even increased slightly  
245 when compared with 48h of treatment. Cells pretreated with BG but without MNU  
246 incubation did not show any increase of DNA damage during the entire period of  
247 treatment.

248

#### 249 *3.2. Cytotoxicity of MNU and BG*

250 The potential cytotoxicity of MNU and BG were evaluated in Caco-2 during the  
251 incubation period. By morphologic observations, none of the treatments resulted in cell  
252 death until the 72h of incubation (data not shown). Induction of cell death by apoptosis  
253 was also evaluated by the TUNEL assay. As shown in Fig. 3, both MNU (500 $\mu$ M) and  
254 BG (100 $\mu$ M), alone or in combination, did not increase the number of apoptotic cells in  
255 Caco-2 until 72h of incubation. However, when Caco-2 cells were pretreated with BG

256 followed by MNU treatment the number of apoptotic cells increased significantly at  
257 96h. This effect is in agreement with the DNA damage observed at 72h when cells were  
258 co-incubated with BG and MNU, due to the recognition of unrepaired O<sup>6</sup>meG base  
259 damage by the MMR system, introduction of SB that lead to cell death.

260

### 261 *3.3. MGMT protein expression*

262 The repair reaction of MGMT is stoichiometric and leads to autoinactivation, and BG is  
263 a pseudosubstrate for MGMT, that acts as an inhibitor of this enzyme by inducing its  
264 autoinactivation and consequent degradation by the proteasome [33]. The extension of  
265 MGMT inhibition is, therefore, reflected in the decreased expression levels of the  
266 protein.

267 The expression levels of MGMT were evaluated after treatment of Caco-2 cells with  
268 different concentrations of BG (0.05-100 µM) for 72h of incubation. As shown in Fig.  
269 4, MGMT protein levels decreased with increasing concentrations of BG. Cells treated  
270 with 0.5 µM BG showed a decrease of around 50% in MGMT protein levels, while in  
271 cells treated with 100 µM of BG only around 5% of the control MGMT levels were  
272 present.

273 To test if MGMT was still inactivated by BG after 72h of incubation under the  
274 experimental conditions used for comet assay, the levels of this protein were evaluated  
275 by western blot. As shown in Fig. 5, BG (100 µM) was able to significantly decrease the  
276 levels of MGMT during the entire experimental period, independently of the presence  
277 of MNU. When Caco-2 cells were incubated with MNU alone, a slight decrease was  
278 observed in MGMT protein expression likely due to MGMT being used to repair  
279 O<sup>6</sup>meG induced by MNU.

280

### 281 *3.4. Concentration-dependent effect of BG on MNU-induced O<sup>6</sup>meG levels in Caco-2* 282 *cells assessed by the comet assay*

283 To test if the concentration-dependent effect of BG on MGMT protein levels correlated  
284 with the accumulation of O<sup>6</sup>meG induced by MNU measured by the comet assay, Caco-  
285 2 cells were incubated with different concentrations of BG (0-100µM) before MNU  
286 addition, and DNA damage was measured after 72h. Again, one hour after incubation  
287 with MNU DNA damage increased in comparison with the control (cells without  
288 MNU), but was completely repaired after 72h of incubation in cells without pre-  
289 treatment with BG (Fig. 6). When Caco-2 cells were pre-treated with different

290 concentrations of BG followed by exposure to MNU, a concentration-dependent  
291 increase in DNA damage at 72h was observed, which was significant for 100 $\mu$ M BG  
292 (Fig. 6). This means that the concentration of 100 $\mu$ M was enough to totally inactivate  
293 cellular MGMT in Caco-2 and the unrepaired O<sup>6</sup>meG lesions converted by MMR in SB  
294 were detectable by the comet assay.

295

### 296 *3.5. Assessment of MNU-induced O<sup>6</sup>meG levels by the comet assay in MMR-deficient* 297 *cells*

298 To prove the involvement of MMR system in the recognition of O<sup>6</sup>meG and consequent  
299 introduction of SB that are detectable by comet assay, HCT116, a MMR deficient colon  
300 cell line, was used. As in Caco-2, after one hour of incubation with MNU initial DNA  
301 damages were detected in HCT116 and 72hr after they were totally repaired (fig 7a).  
302 However, no differences were observed between cells with and without BG treatment  
303 after 72hr of MNU incubation. In MMR deficient cells therefore, inhibition of MGMT  
304 does not increase SB.

305 In the TUNEL assay (fig 7b), both MNU (500 $\mu$ M) and BG (100 $\mu$ M), alone or in  
306 combination, did not increase the number of apoptotic cells in HCT116 cells.

307

### 308 *3.6. Assessment of the effects of MMS by the comet assay in MMR-proficient cells*

309 To test if our system is specific for O<sup>6</sup>meG, MMS, an alkylating agent that does not  
310 induce O<sup>6</sup>meG lesions in significant amounts was used. Caco-2 cells were treated with  
311 MMS in the presence or absence of the BG. DNA damage was assessed by the comet  
312 assay after different times of incubation with MMS such as in MNU treatment. As  
313 shown in Fig. 8, one hour after incubation with MMS, significant DNA damage was  
314 detected in Caco-2 cells. After 72hr of incubation, the levels of DNA damage decreased  
315 until control values without MMS treatment were reached. In the presence of BG, DNA  
316 damage repair was similar to control because MMS induces very low levels of O<sup>6</sup>meG  
317 (almost nonexistent). Due to the absence of O<sup>6</sup>meG induction by MMS, the damages are  
318 not converted to SB by MMR and therefore nothing is detected by the comet assay.

319

## 320 **4. Discussion**

321 Here we report that the comet assay can be used to assess the levels of a specific  
322 alkylating DNA damage, O<sup>6</sup>meG, due to the fact that these lesions are converted to SB  
323 by proliferating, MMR proficient cells when MGMT is inhibited. Caco-2 cells were

324 treated with MNU and significant DNA damage (such as SB and AP sites) was  
325 observed by the comet assay after 1h of incubation. Most of the damage was totally  
326 repaired at the time of the second round of replication in Caco-2 cells. However, when  
327 Caco-2 cells were also incubated with BG, the inhibitor of MGMT, significantly higher  
328 DNA damage was present at 72h. MGMT is a suicide repair enzyme responsible for  
329 O<sup>6</sup>meG repair. If the enzyme does not repair all O<sup>6</sup>meG lesions, O<sup>6</sup>meG:T mismatches  
330 will be generated upon cell division. This new damage is recognized by the proteins of  
331 the MMR pathway that, in attempting to repair the mismatch, generate SB in the DNA.  
332 As long as O<sup>6</sup>meG remains in one of the DNA template strands, the MMR repair  
333 process will be repeated, creating a “futile repair loop” that results in double-strand  
334 breaks. Accumulation of SB will eventually induce cell death by apoptosis.

335 In this study, BG was used at a concentration that inhibited MGMT in Caco-2 cells, as  
336 shown by western blot. When the concentration of BG was decreased, the expression of  
337 MGMT at 72h was higher in a BG concentration-dependent manner. This effect was  
338 reflected in the comet assay results, where increasing concentrations of BG resulted in  
339 increasing DNA damage at 72h, showing that detected SB in the presence of BG result  
340 from unrepaired O<sup>6</sup>meG base damages due to inhibition of MGMT.

341 In agreement with this, in Caco-2 cells co-treated with BG and MNU the number of  
342 apoptotic cells increased after 96h of incubation. This is due to the accumulation of SB  
343 introduced by the action of the MMR system on O<sup>6</sup>-meG:T mismatch (since O<sup>6</sup>meG  
344 still remains in the DNA template). SB accumulate and become cytotoxic after some  
345 replication rounds inducing cell death by apoptosis. Treatment with MNU or BG alone  
346 did not induce apoptosis, since in the first case O<sup>6</sup>meG is repaired by MGMT and in the  
347 second, in spite of the almost total inhibition of MGMT, the basal levels of O<sup>6</sup>meG are  
348 negligible and not enough to produce SB and induce apoptosis.

349 In order to test the specificity of this modified comet to detect SB originated by MMR  
350 action on O<sup>6</sup>meG, a MMR deficient cell line (HCT116) was used. As expected, in  
351 HCT116 cells, DNA damage remains similar to basal levels even in the presence of BG.

352 In MMR deficient cells the comet assay will not detect O<sup>6</sup>meG damages because they  
353 will not be transformed into SB and therefore will be undetectable by the comet assay.

354 In accordance with the comet assay results, in the MMR deficient HCT116 cells,  
355 apoptosis did not occur with co-treatment with MNU and BG, since O<sup>6</sup>-meG:T  
356 mismatches are not recognized in these cells, SB are not produced and therefore no  
357 apoptosis takes place.

358 As referred previously, MNU also induces N-alkylation lesions that are repaired by  
359 BER pathway. The N-methylpurine-DNA glycosylase (MPG), a glycosylase of BER  
360 pathway, initiate N-alkylation repair by hydrolysis of the N-glycosylic bond creating an  
361 AP site that is repaired by the other enzymes of the BER pathway [5, 34]. To test the  
362 possibility of N-alkylations involvement on the detected SB in the presence of BG,  
363 Caco-2 cells were treated with an inhibitor of BER pathway, methoxyamine (Mx). Mx  
364 inhibits MPG resulting in AP sites accumulation, a type of lesion that is usually detected  
365 by the comet assay. However, at 72h no increase of DNA damage was detected when  
366 cells were treated with Mx (data not shown), which means that N-alkylations were  
367 repaired thereby not contributing for the increase of DNA damage detected in the  
368 presence of BG. The use of Mx demonstrates that the assay is detecting O<sup>6</sup>meG only.  
369 Further confirmation is provided by the use of a second alkylating drug, MMS, which  
370 although inducing N-alkylations virtually does not produce O<sup>6</sup>meG lesions, and no  
371 damage was detected by our system. Therefore in order to assess by comet assay  
372 (CoMeth) O<sup>6</sup>meG levels and infer about MGMT activity we propose the use of  
373 proliferative and MMR proficient cells, such as Caco-2 cells. The assay include several  
374 steps: First, cells should be pretreated with several concentrations of BG, selecting a  
375 concentration sufficient to inhibit all MGMT protein of the cells. Second, cells should  
376 be exposed to the alkylating agent and DNA damage assessed at least at three different  
377 times, 1, 24 and 72h (these points should be chosen according to the doubling time of  
378 the cells). Third, cells without BG treatment should be included as a control. The initial  
379 DNA damages (if present) are repaired over time and, at 72h, in the absence of  
380 pretreatment with BG, the levels of DNA damage should reach control values.  
381 However, in cells pretreated with BG followed by the alkylating agent an increase of  
382 DNA damage will be observed over time. This increase corresponds to the conversion  
383 of O<sup>6</sup>meG into SB by MMR pathway. The difference between DNA damage in cells  
384 with and without pretreatment with BG, reflect O<sup>6</sup>meG levels induced by the alkylating  
385 agent (Fig. 9). This new application of comet assay allows qualitatively assessment of  
386 O<sup>6</sup>meG levels. However, because inhibition reaction of MGMT by BG is a  
387 stoichiometric reaction (1:1), the molar concentration of BG used to inhibited MGMT  
388 allows to infer the amount of MGMT active present in the cells. New advancements in  
389 the comet assay, such as the one described or the recent high throughput applications  
390 such as GelBond film [35], specific 96-well plates [36, 37], glass microscope slides  
391 [38][39] and micro cell arrays [40][41] make the comet assay an attractive method for

392 compound screening. In conclusion, we demonstrate that the comet assay can be used  
393 also to assess alkylating DNA damage, specifically O<sup>6</sup>meG, induced in proliferating and  
394 MMR efficient cells, by the use of a specific inhibitor of MGMT. This new application  
395 (CoMeth) allows the study of new MGMT inhibitors, the test of potential  
396 chemopreventive and chemotherapeutic drugs that act by modulating the activity of  
397 MGMT or O<sup>6</sup>meG levels produced, respectively, as well as drugs that increase  
398 expression by demethylation of silenced MGMT or MMR genes. With this new  
399 application to the study of alkylating agents we expect to contribute to the widespread  
400 use of the comet assay.

401 Conflict of interest

402 There are no conflicts of interest to report.

403

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523  
524



## Figure legends

Figure 1. Cell fate and repair of DNA damage induced by methylating agents involving MGMT and MMR pathways.

Figure 2. DNA damage induced by MNU in the presence and absence of BG in Caco-2 cells, as assessed by the comet assay. Cells were pre-treated without or with BG (100 $\mu$ M) (white and black bars, respectively) for 2h before MNU (500 $\mu$ M) treatment during 1, 24, 48 and 72h. Results are expressed as mean  $\pm$  SEM at least three independent experiments. Significant differences (\*  $P < 0.05$ ) when compared with cells without BG treatment at 72h were determined by *t*-test.

Figure 3. Effect of MNU (500 $\mu$ M), BG (100 $\mu$ M) and BG plus MNU on apoptosis induction in Caco-2 cells. Apoptosis was measured by the TUNEL assay after 48, 72 and 96h of incubation with the compounds. Results are expressed as mean  $\pm$  SEM at least three independent experiments. Significant differences (\*\*\*)  $P < 0.001$  when compared with the respective control were determined by *t*-test.

Figure 4. Effect of different concentrations of BG (0.05-100 $\mu$ M) on MGMT levels in Caco-2 cells after 72h of incubation, assessed by western blot. Actin antibody was used as a loading control. Results are expressed as mean  $\pm$  SEM of at least three independent experiments. Significant differences (\*  $P < 0.05$  and \*\*\*  $P < 0.001$ ) when compared with the control were determined by One-way ANOVA followed by Newman-Keuls Multiple comparison test.

Figure 5. Effect of MNU (500 $\mu$ M), BG (100 $\mu$ M) and BG plus MNU on MGMT protein expression in Caco-2 cells. MGMT protein expression was measured after 24, 48 and 72 of incubation with the compounds by western blot. The blot image is representative of the effect observed in three independent experiments.

Figure 6. Effect of different concentrations of BG (0.1-100 $\mu$ M) on MNU-induced O<sup>6</sup>meG levels in Caco-2 cells as assessed by the comet assay. Cells were pre-treated with BG for 2h followed by MNU (500 $\mu$ M) treatment for 1 (grey bars) or 72h (white bars). Results are expressed as mean  $\pm$  SEM at least three independent experiments. Significant differences (\*\*\*)  $P < 0.001$  when compared with the respective control were

determined by One-way ANOVA followed by Newman-Keuls Multiple comparison test.

Figure 7. Effect of MNU on DNA damage, assessed by the comet assay (A) and on apoptosis induction, assessed by TUNEL assay (B) in HCT116 cells, in the presence and absence of BG. Cells were pre-treated without or with BG (100 $\mu$ M) (white and black bars, respectively) for 2h before MNU (500 $\mu$ M) treatment during 1, 24, 48 and 72h for comet assay and during 48, 72 and 96h for TUNEL assay. Results are expressed as mean  $\pm$  SEM at least three independent experiments.

Figure 8. DNA damage induced by MMS in Caco-2 cells in the presence and absence of BG, as assessed by the comet assay. Cells were pre-treated with BG (0, 1, 10 and 100 $\mu$ M) for 2h before MMS (200 $\mu$ M) treatment during 1 and 72h. Results are expressed as mean  $\pm$  SEM at least three independent experiments.

Figure 9. Detection of O<sup>6</sup>meG DNA damage induced by alkylating agents in MMR proficient cells using an inhibitor of MGMT by comet assay. Representation of possible results and interpretation.

Figure 1  
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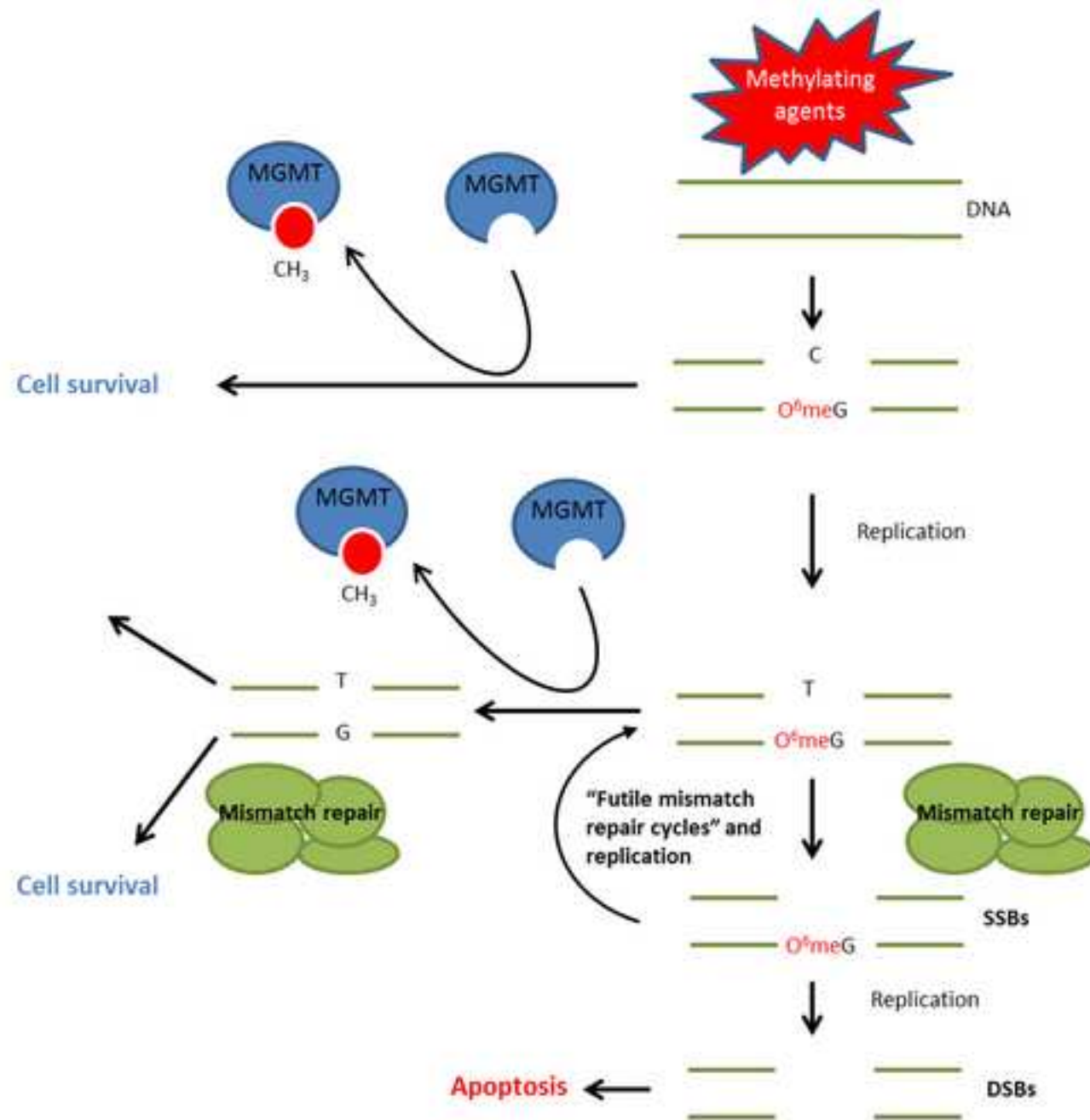


Figure 2  
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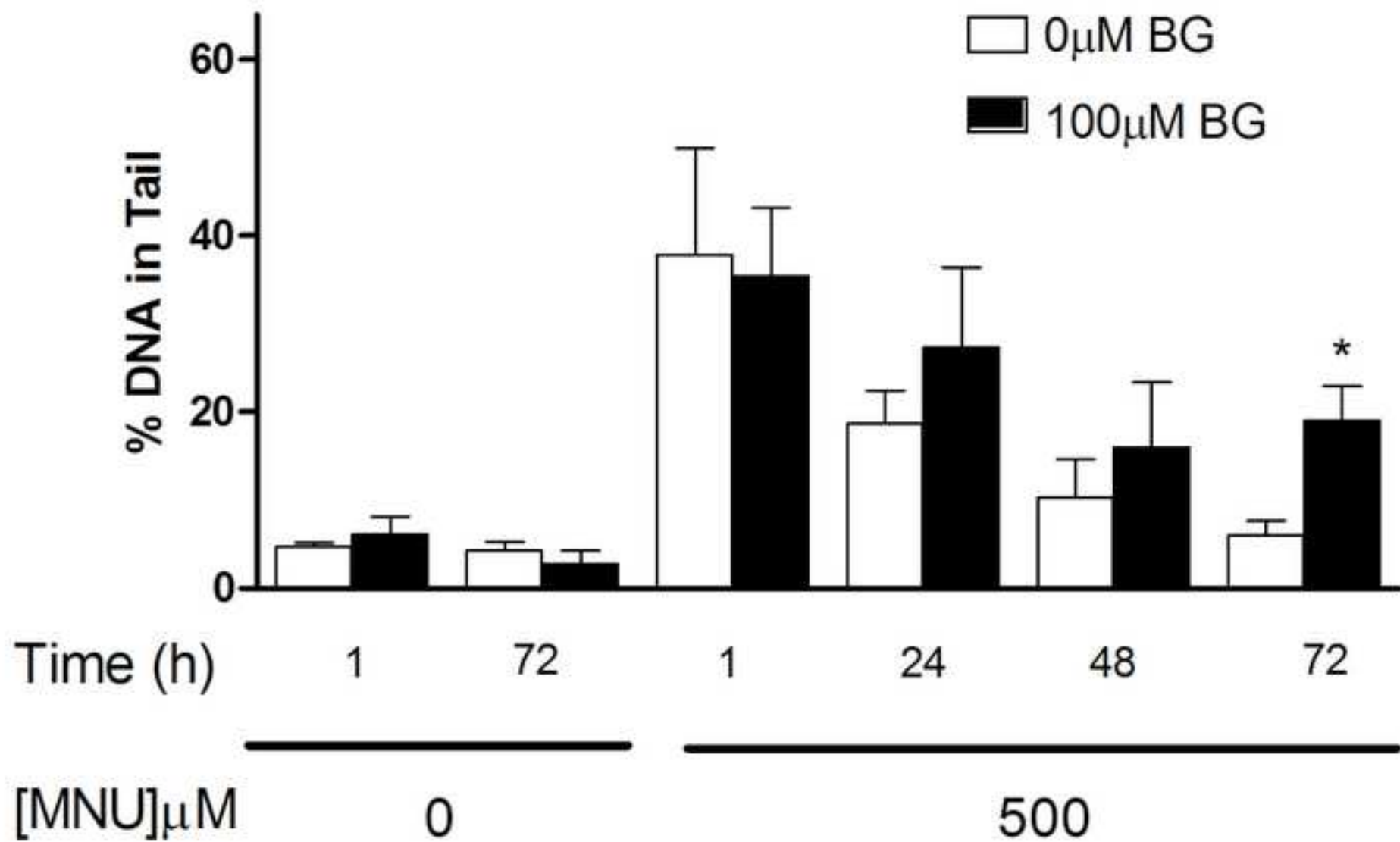


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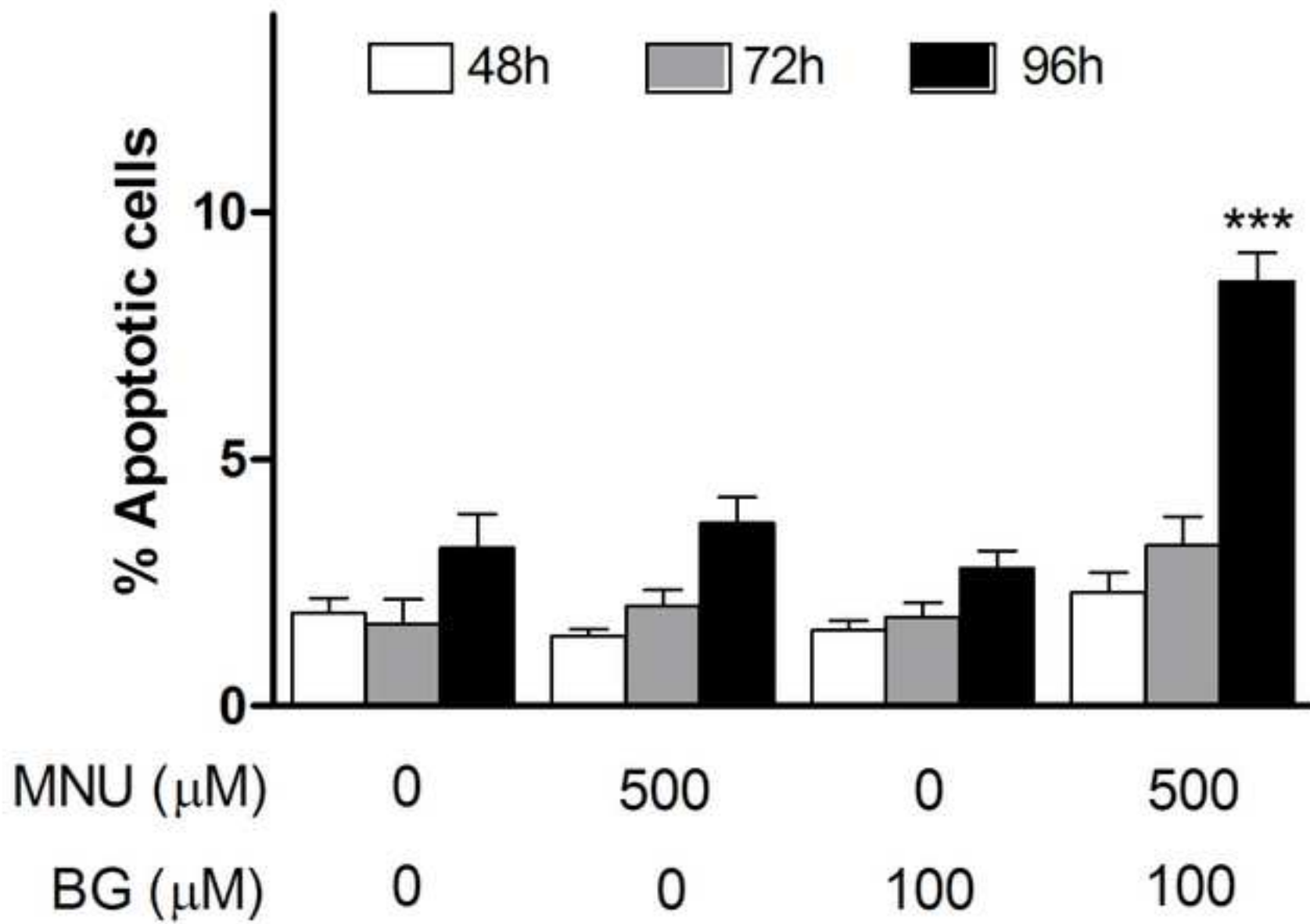


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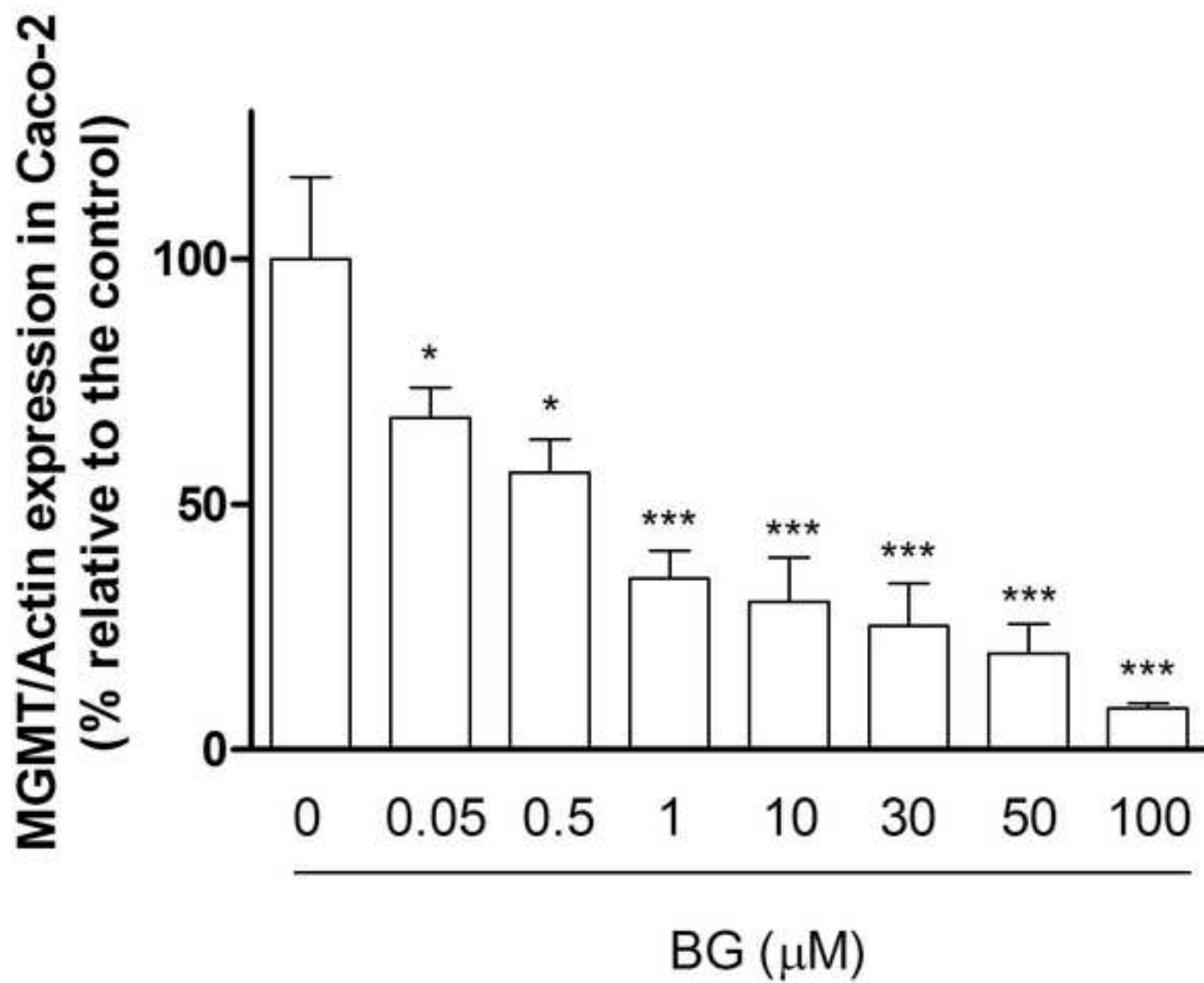


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## MGMT – Caco-2

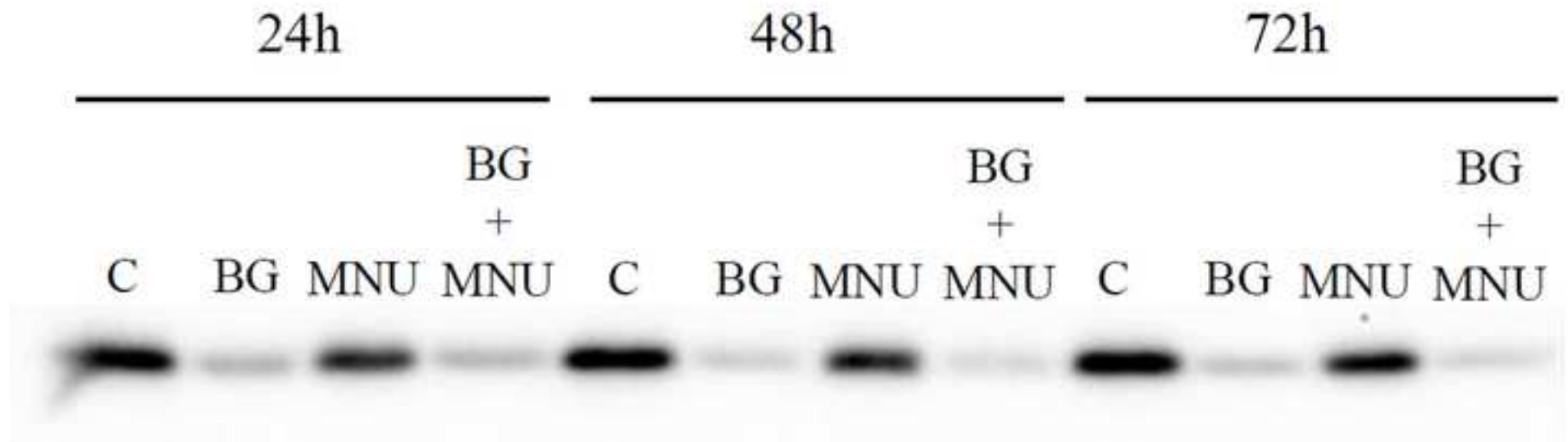
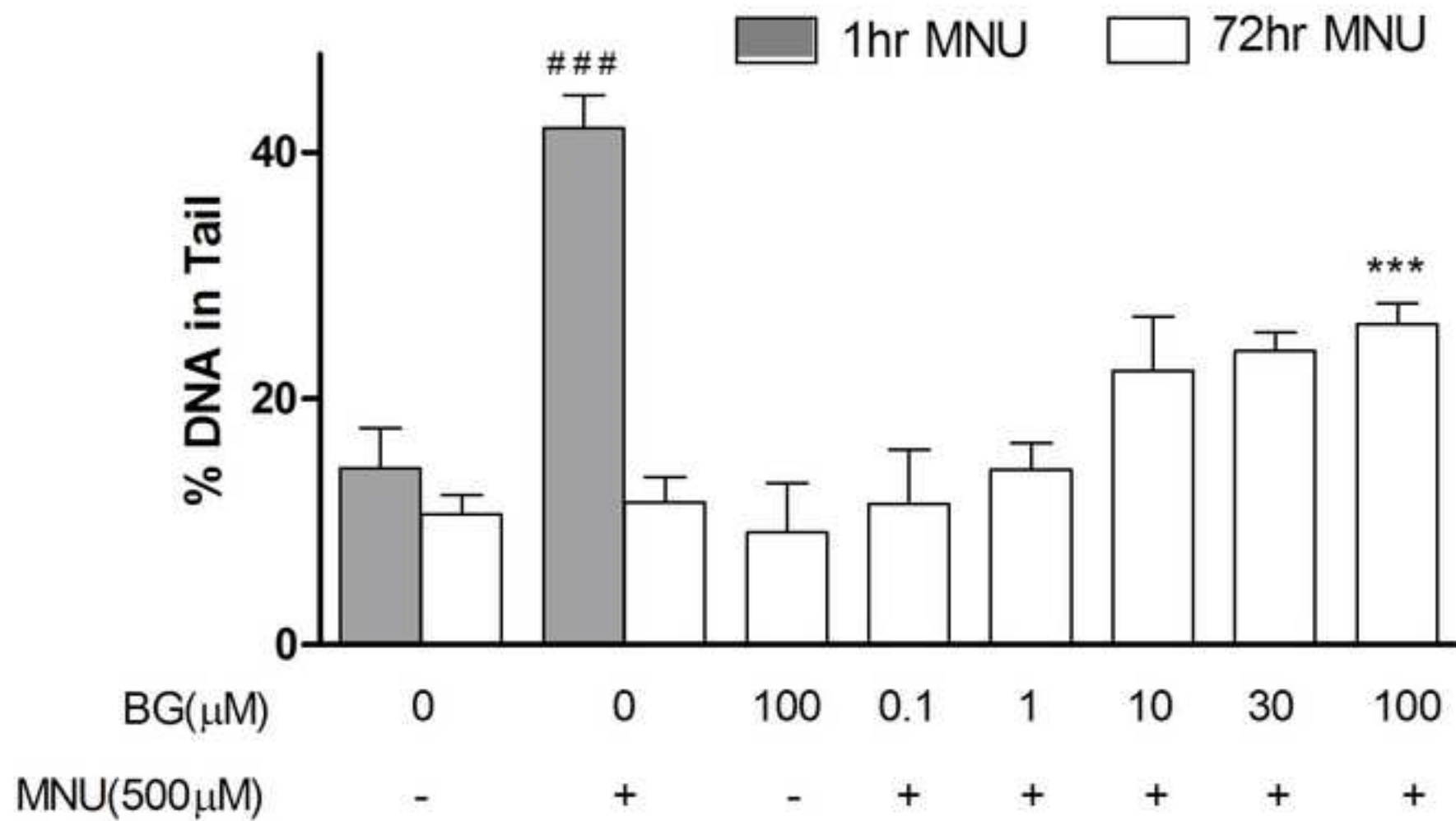
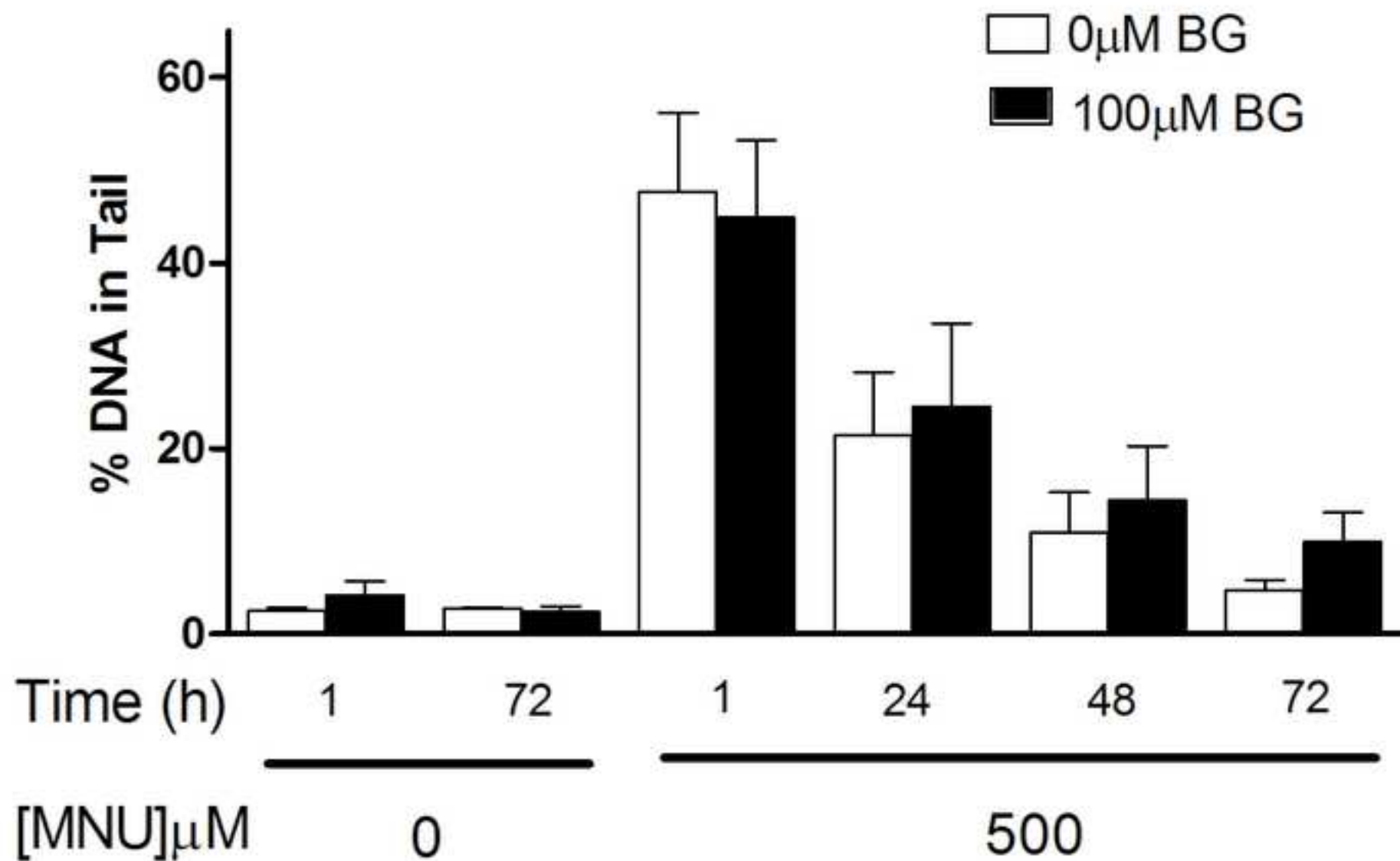


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### (A) Comet assay



## (B) TUNEL assay

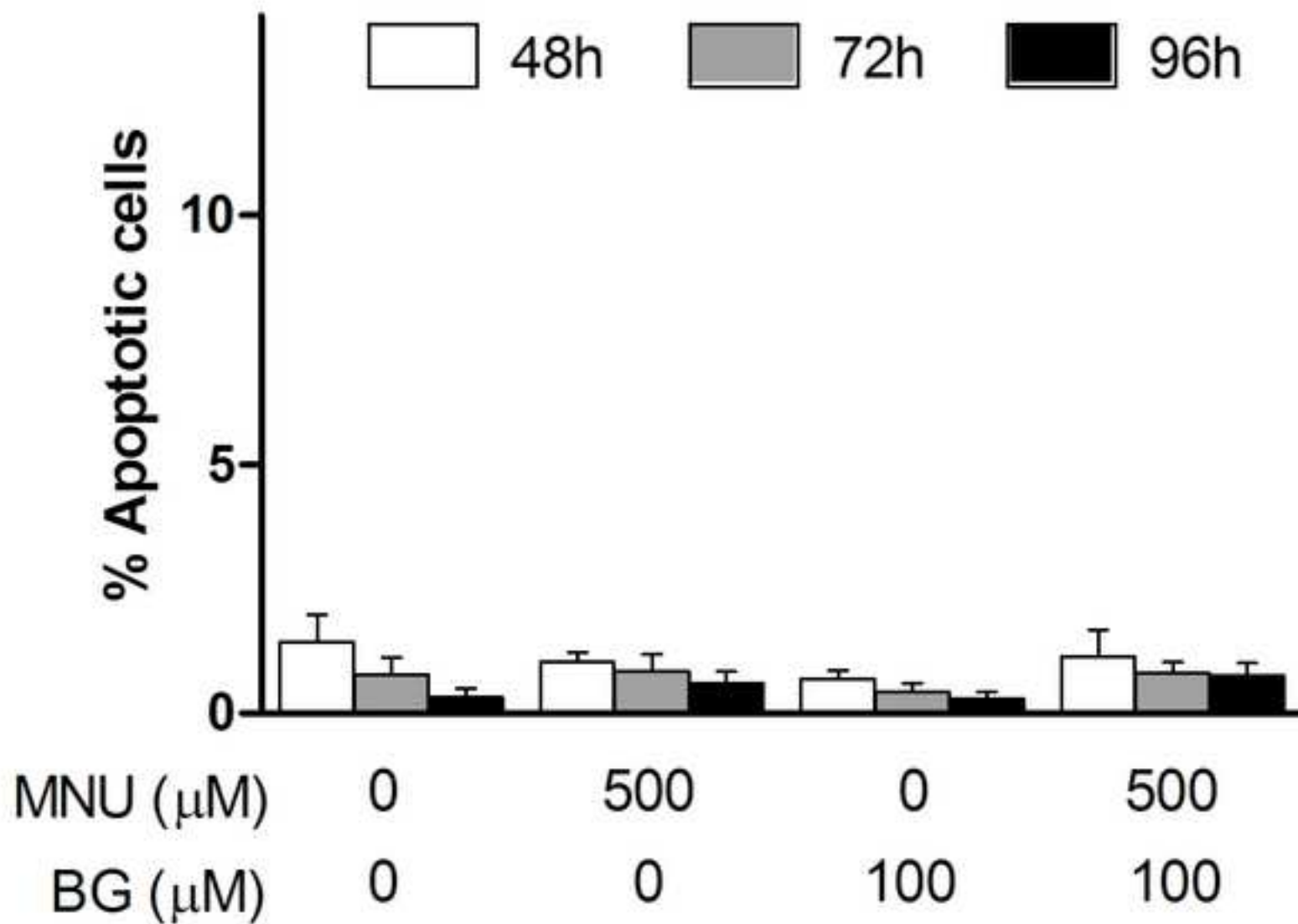


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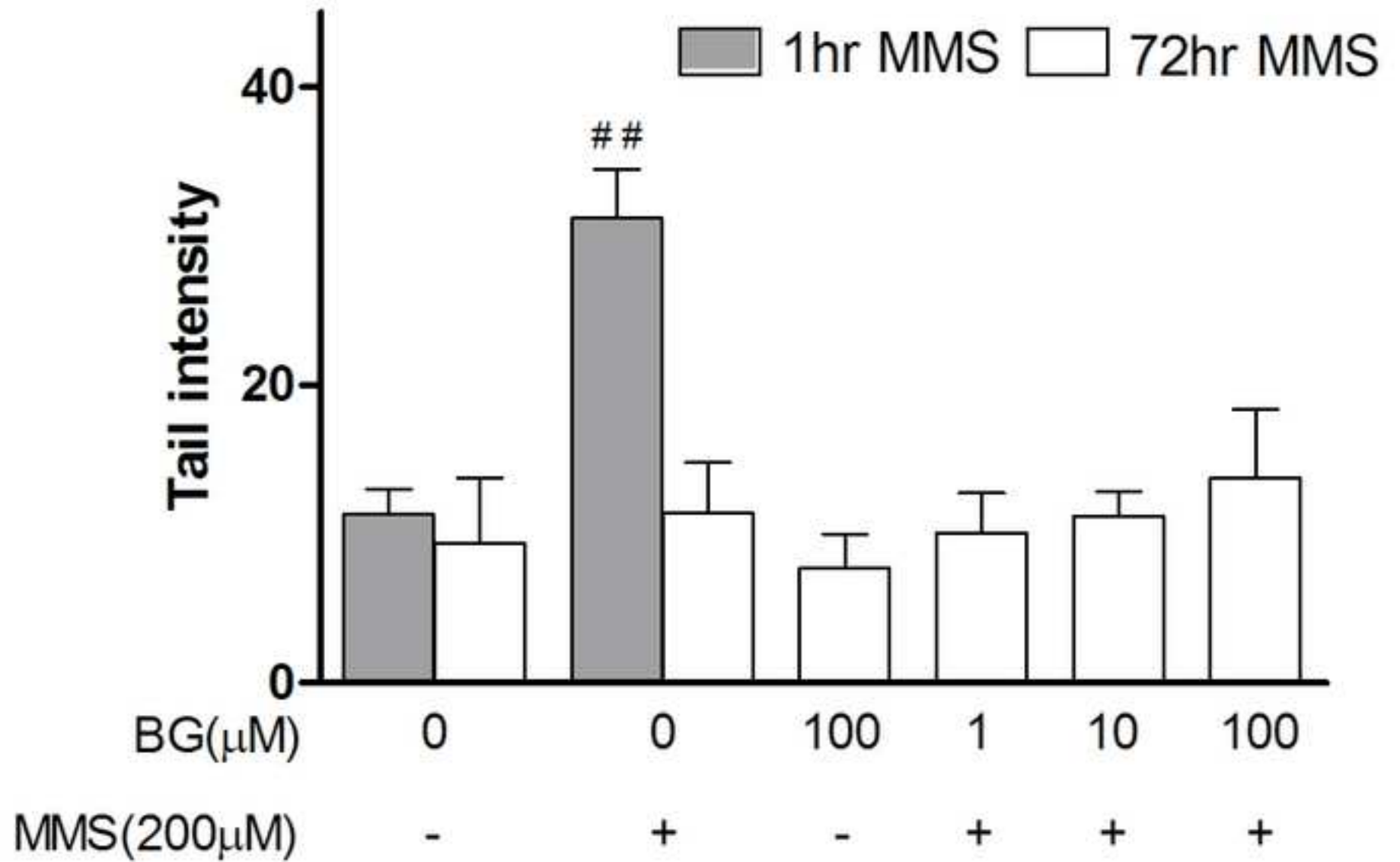


Figure 9  
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