Development of a new application of the comet assay to assess levels of O^6-methylguanine in genomic DNA (CoMeth)

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Abstract

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

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

Keywords: comet assay, O^6meG lesion, MGMT, MMR system, alkylating chemotherapy

Abbreviations: Base excision repair (BER); O^6-benzylguanine (BG); Methoxyamine (Mx); Methyl methanesulfonate (MMS); Mismatch repair (MMR); N-methyl-N-nitrosourea (MNU);

N-methylpurine-DNA glycosylase (MPG); O^6-methylguamine (O^6meG); O^6-methylguanine-methyltransferase (MGMT); Strand breaks (SB).

1. Introduction

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

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

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

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

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

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

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

chemotherapeutic drugs and/or chemopreventive agents on MGMT activity that also allow to screen the effects on silencing of MGMT or MMR by promoter methylation.

Single cell gel electrophoresis (SCGE) assay or the comet assay is a simple, fast and low cost method to assess DNA damage [25, 26]. The comet assay is widely used to measure oxidatively damaged DNA where it allows the evaluation of effects of environmental factors and chemoprevention by dietary constituents [27, 28]. Alkylating DNA damage occurs more frequently than oxidative damage and is also a more relevant driver of mutagenesis or inducer of cell death in chemotherapy. Beside oxidant agents, comet assay has been also used to assess strand breaks induced by alkylating agents [29, 30]. However, its application to estimate the levels of O^6meG bases has not been explored. In the present study we modified the comet assay in order to enable it to assess O^6meG, after its conversion into SB in MGMT inactivated and MMR proficient cells. We demonstrate that by this O^6methylation specific variant of the comet assay – CoMeth assay- O^6meG levels can be assessed, in proliferating and MMR proficient cells.

2. Materials and methods

2.1. *Chemicals*

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

2.2. *Cell culture*

Caco-2 cells (derived from human colon carcinoma) and HCT116 cells were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and antibiotics (100U/ml penicillin and 100µg/ml streptomycin), under an atmosphere of 5% CO_2 at 37ºC. Cells were trypsinized when nearly confluent.

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

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

2.3. *Apoptosis assay*

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

2.4. *MGMT protein expression*

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

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

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

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

After 24, 48 and 72h of incubation of Caco-2 or HCT116 cells with MNU (with or without BG) DNA damage was assessed by the comet assay as previously described [28, 32]. Briefly, after treatment, Caco-2 and HCT116 cells were trypsinized, washed, centrifuged, and the pellet suspended in low melting point agarose; about $2x10^4$ cells were placed on a slide (pre-coated with 1% normal melting point agarose and dried) and covered with a coverslip. After 10 min at 4 ºC, the coverslips were removed and slides were placed in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris Base, pH 10 plus 1% Triton X-100) for 1h at 4ºC. Slides were then placed in a horizontal electrophoresis chamber with electrophoresis solution (300 mM NaOH, 1 mM Na_2EDTA, pH >13) for 40 min at 4ºC for the DNA to unwind before electrophoresis was run for 20 min at 0,8V/cm and ~300 mA. After electrophoresis, slides were washed two times with PBS and dried at room temperature. For analysis of the comet images, slides were stained with SYBR Gold solution for 30 min at 4ºC; after drying, slides were analyzed in a fluorescence microscope and Comet IV analysis system (Perceptive Instruments Ltd, Haverhill, UK) was used to calculate the parameter % of DNA in tail. About 100 randomly selected cells were analyzed per sample.

2.5.1. *Effect of BG on O^6meG levels in Caco-2 cells assessed by the comet assay*

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

2.5.2. Assessment of MMS-induced O^6meG levels by the comet assay in MMR-proficient cells

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

2.6. *Statistical analysis*

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

3. Results

3.1. *Assessment of MNU-induced O^6meG levels by the comet assay in MMR efficient cells*

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

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

3.2. Cytotoxicity of MNU and BG

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

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

3.3. MGMT protein expression

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

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

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

3.4. Concentration-dependent effect of BG on MNU-induced O^6meG levels in Caco-2 cells assessed by the comet assay

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

concentrations of BG followed by exposure to MNU, a concentration-dependent increase in DNA damage at 72h was observed, which was significant for 100μM BG (Fig. 6). This means that the concentration of 100μM was enough to totally inactivate cellular MGMT in Caco-2 and the unrepaired O^6meG lesions converted by MMR in SB were detectable by the comet assay.

3.5. Assessment of MNU-induced O^6meG levels by the comet assay in MMR-deficient cells

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

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

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

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

4. Discussion

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

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

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

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

In order to test the specificity of this modified comet to detect SB originated by MMR action on O^6meG, a MMR deficient cell line (HCT116) was used. As expected, in HCT116 cells, DNA damage remains similar to basal levels even in the presence of BG. In MMR deficient cells the comet assay will not detect O^6meG damages because they will not be transformed into SB and therefore will be undetectable by the comet assay. In accordance with the comet assay results, in the MMR deficient HCT116 cells, apoptosis did not occur with co-treatment with MNU and BG, since O^6-meG:T mismatches are not recognized in these cells, SB are not produced and therefore no apoptosis takes place.

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

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

Conflict of interest

There are no conflicts of interest to report.

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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 (100µM) (white and black bars, respectively) for 2h before MNU (500µM) treatment during 1, 24, 48 and 72h. Results are expressed as mean ± 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 (500µM), BG (100µM) 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 ± 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 (0.05-100µM) 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 ± 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 (500µM), BG (100µM) 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 (0.1-100µM) on MNU-induced O^6meG levels in Caco-2 cells as assessed by the comet assay. Cells were pre-treated with BG for 2h followed by MNU (500µM) treatment for 1 (grey bars) or 72h (white bars). Results are expressed as mean ± 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 (100µM) (white and black bars, respectively) for 2h before MNU (500µM) treatment during 1, 24, 48 and 72h for comet assay and during 48, 72 and 96h for TUNEL assay. Results are expressed as mean ± 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 100µM) for 2h before MMS (200µM) treatment during 1 and 72h. Results are expressed as mean ± SEM at least three independent experiments.

Figure 9. Detection of O^6meG 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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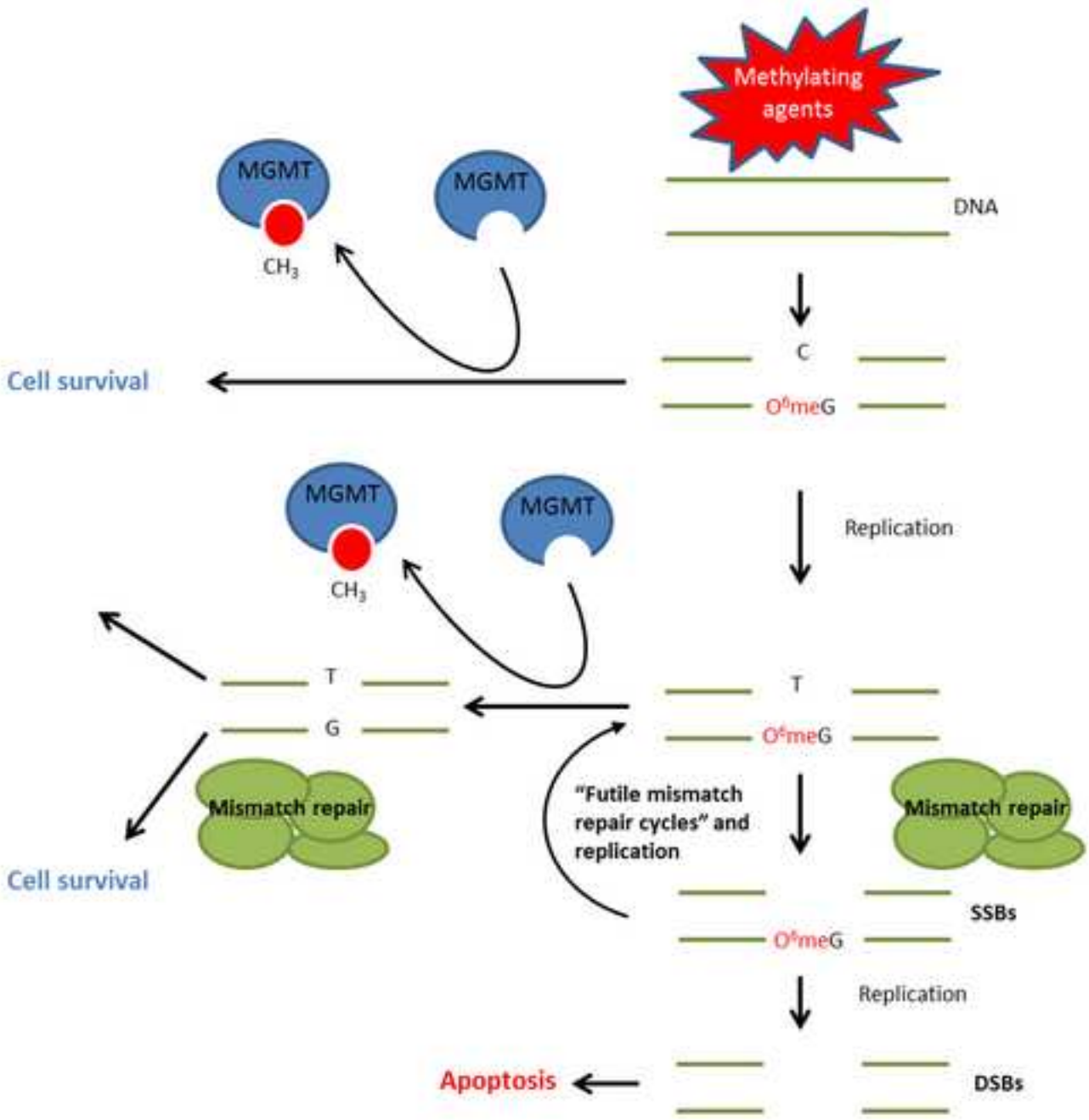

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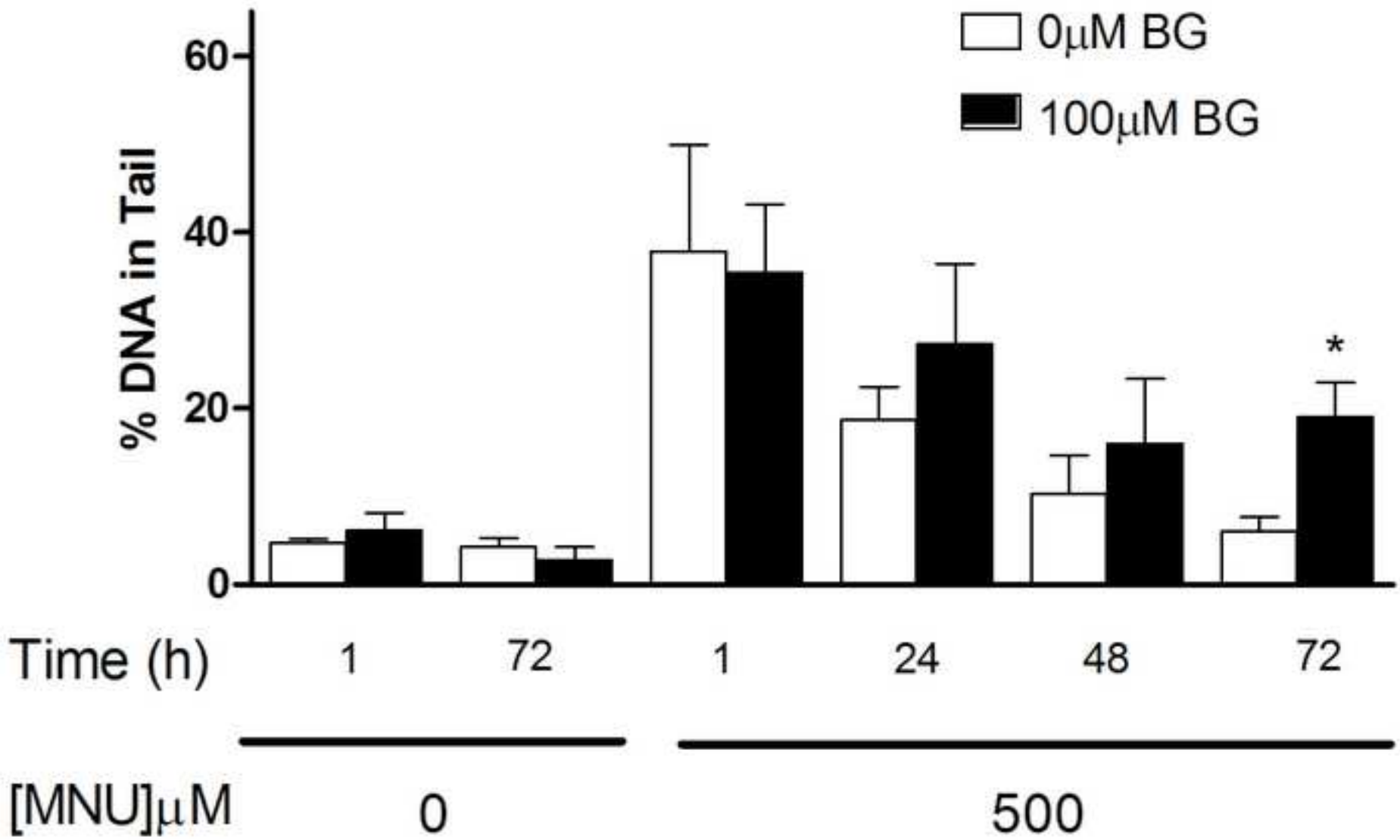

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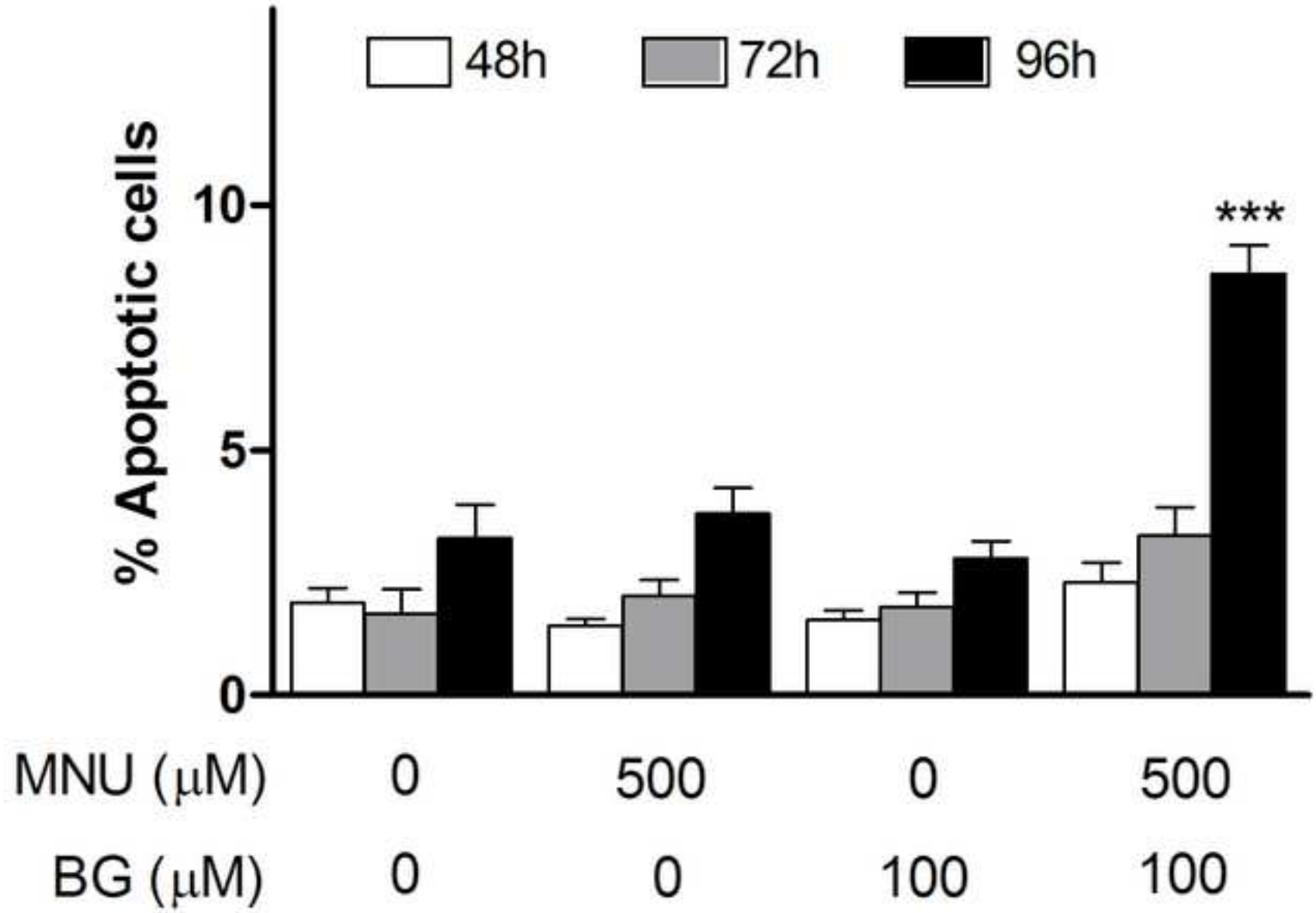

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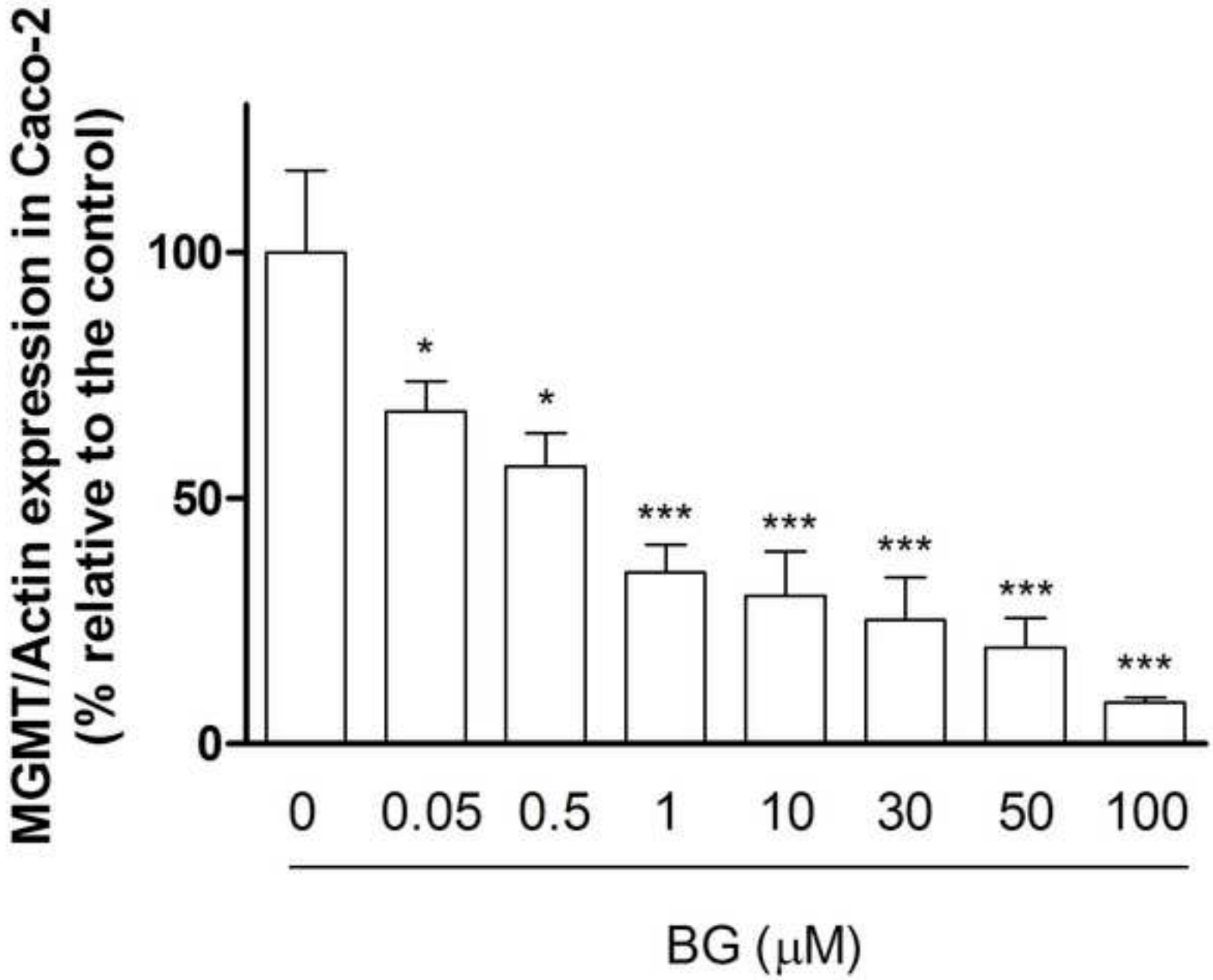

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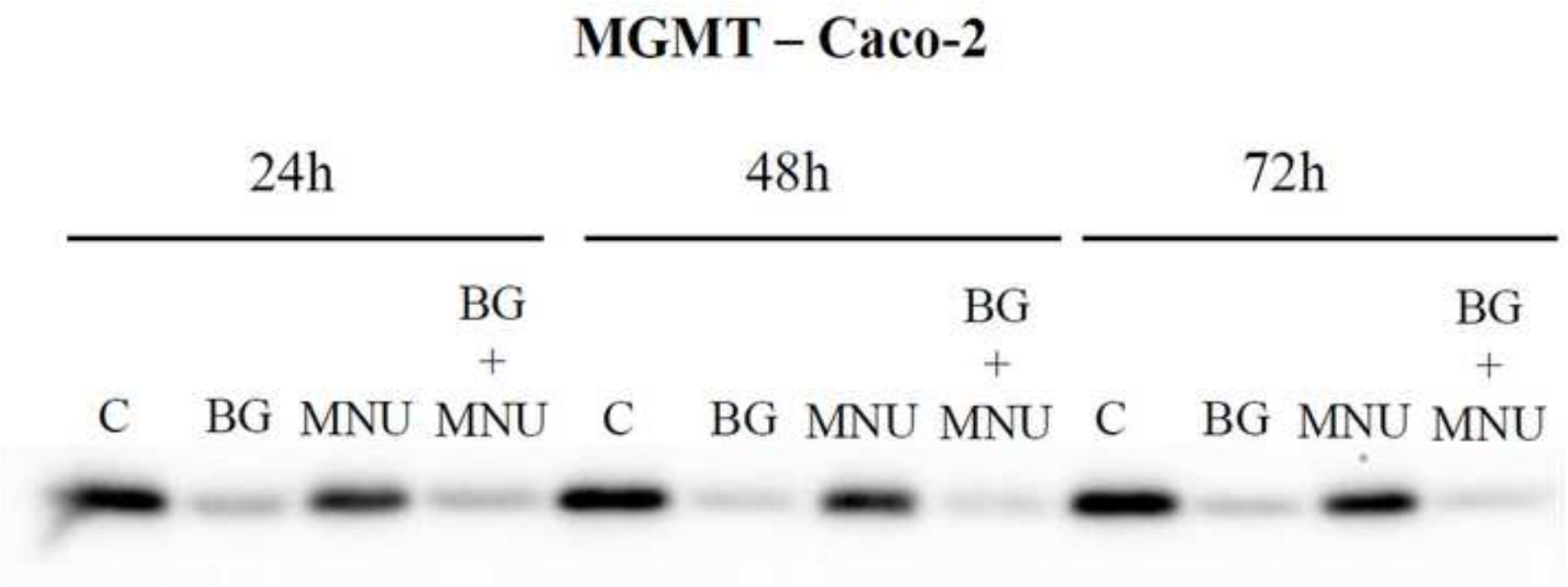

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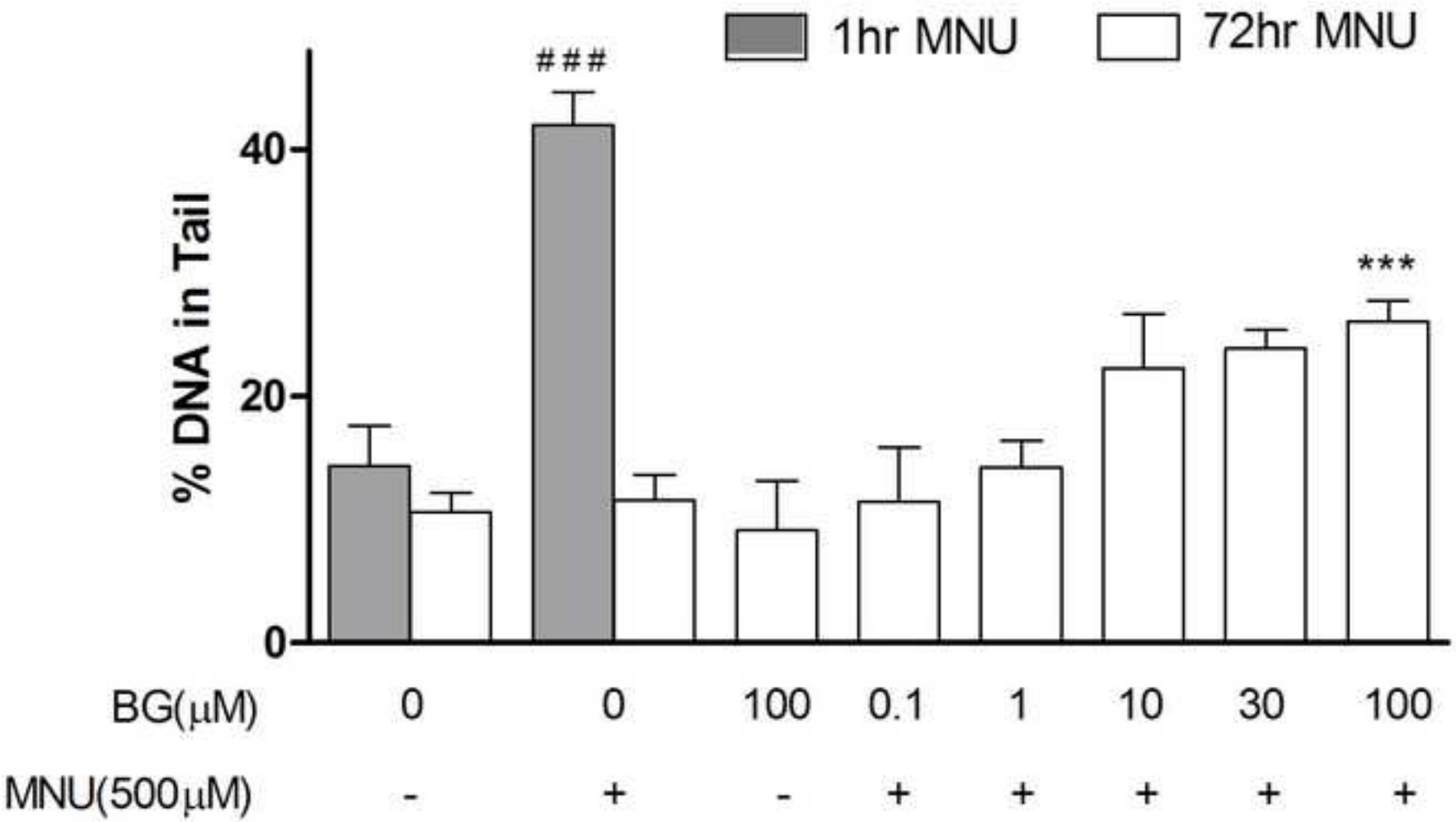

Figure 7A
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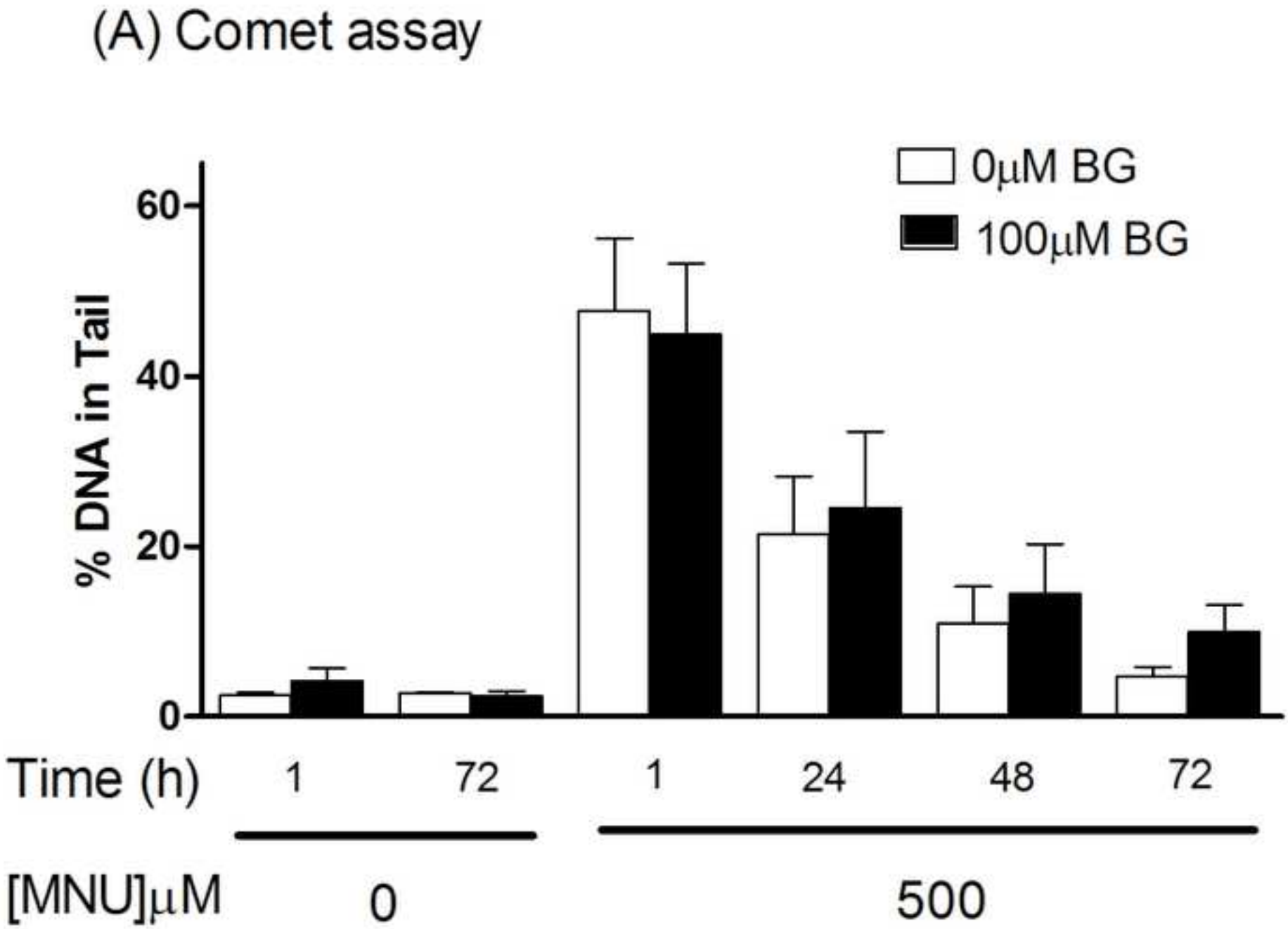

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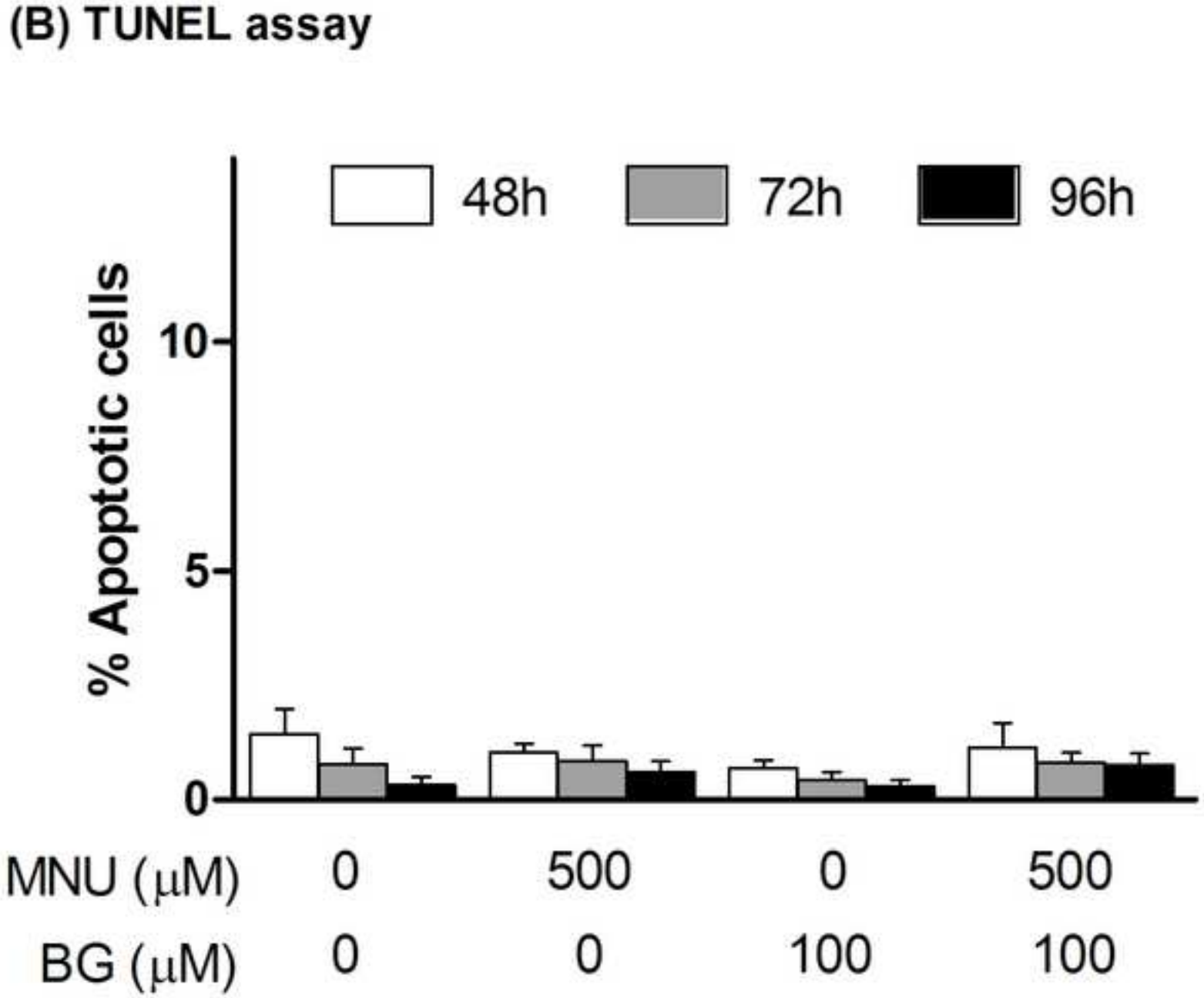

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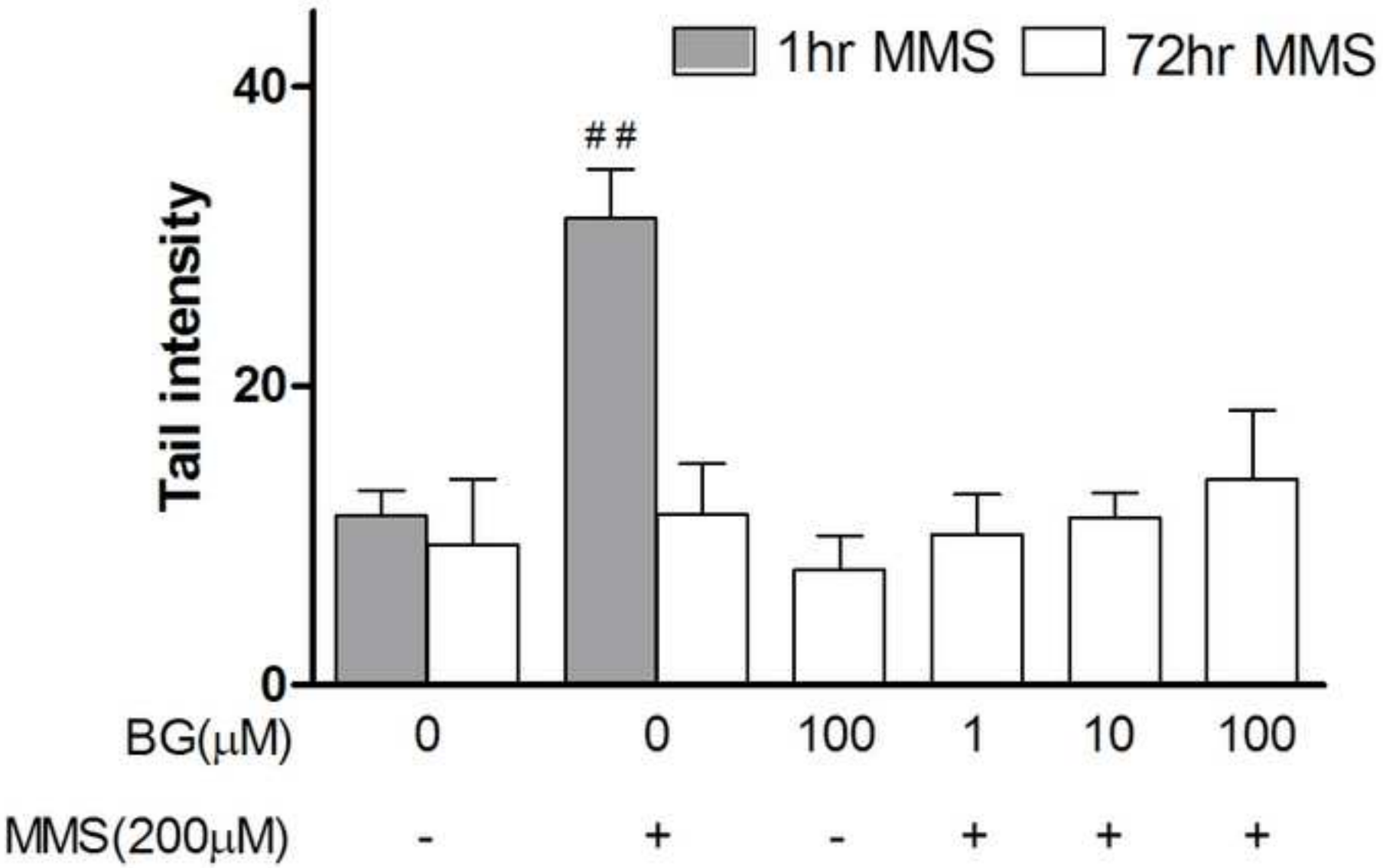

Figure 9

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