Earthworms Repair H_2O_2-Induced Oxidative DNA Adducts without Removing UV-Induced Pyrimidine Dimers

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Abstract. Ultraviolet (UV) radiation is a natural insult to various organisms. Earthworms, although possessing similar biomolecules to those in mammalian skin, do not suffer from skin cancer nor any other types of cancer as humans do. However, little is known about the molecular mechanism of the earthworm's tolerance to UV. In this study, we evaluated the genotoxicity of UV and the capacity of earthworm cell to repair UV-induced damage. The T4 UV endonuclease UV-incorporated comet assay was used to examine the excision and rejoining steps of UV-induced pyrimidine dimer. Earthworm testis cells were treated with a combination of 5 mM hydroxyurea plus 50 μM cytosine-β-D-arabinofuranoside for 6 h to block DNA rejoining capacity and to investigate excision dynamics. Compared with H_2O_2-induced oxidative repair capacity, the excision step of repair of UV-induced lesions in earthworm testis cells was significantly lower. After 6-h treatment of 5 mM hydroxyurea plus 50 μM cytosine-β-D-arabinofuranoside, the medium was totally replaced with fresh medium and cells were allowed to rejoin the accumulated DNA strand breaks. We found that the capacity for rejoining UV-induced

breaks was also significantly lower than that for the H_2O_2-induced breaks. Our results strongly suggest that earthworms seem to be efficient at repairing H_2O_2-induced oxidative DNA adducts, but not so capable of removing UV-induced pyrimidine dimers from their genome.

Ultraviolet (UV) light, which is commonly divided into UV-A (320-400 nm), UV-B (280-320 nm), and UV-C (200-280 nm), is one of the components of solar radiation that significantly affects various organisms (1, 2). Among UVA, B and C, UVC is most mutagenic and well analyzed investigated. When DNA is exposed to UVC radiation, adjacent pyrimidines become covalently linked by the formation of a four-membered ring structure that results from saturation of their respective 5,6 double bonds (3). The structure formed by this photochemical cyclo-addition is referred to as a cyclobutane di-pyrimidine or cyclobutane pyrimidine dimer (CPD). Single-stranded DNA also permits the formation of dimers between nonadjacent pyrimidines (4). In humans, exposure to sunlight may cause skin cancer and UV-induced DNA adducts, leading to downstream genome instability which can affect skin carcinogenesis. UVA may alter cell membrane components (5), induce DNA–protein crosslinking (6), and increase formation of reactive oxygen species (ROS). UVC, may also induce production of ROS and DNA adducts, CPDs, which are repaired *via* the nucleotide excision repair (NER) pathway (7).

Usually, earthworms do not expose themselves to sunlight for extended periods. It is suggested that UV radiation is an important factor causing damage to earthworms when they are on the soil surface. Although earthworms possess biomolecules similar to those found in mammalian skin (8), they do not develop skin cancer or any other forms of cancer. Limited literature has provided evidence for UV as being harmful to earthworms (8-10). This study was designed to reveal: i) the unknown mechanisms that define earthworm avoidance of sunlight; (they actually avoid light exposure, except at night when they forage and are confronted by infra-red light); ii) mechanism of cellular DNA repair for UV-induced DNA damage. The details of the mechanism of cellular repair of UV radiation damage in the earthworm are poorly understood.

Materials and Methods

Earthworms and chemicals. The ear thworms (*Metaphire posthuma*) were purchased from an earthworm supply store, maintained in their native soil, and fed with peels of tangerine under dark and moist conditions before their sacrifice. Healthy adult earthworms with a well-developed clitellum were scarified and their testis cells carefully removed for UV radiation

and comet assay.

All chemicals and solvents used throughout this study were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Aldrich Chemical Co. (Milwaukee, WI, USA). T4 UV endonuclease V was purchased from Epicentre Technologies (Madison, WI, USA). Formamidopyrimidine-DNA glycosylases (Fpg) and endonuclease III were purchased from Trevigen (Gaithersburg, MD, USA).

UV density measurement and UV exposure. The UV light crosslinker (Spectrolinker XL-1000, Spectronics Co., Westburg, NY, USA) was used; and the UV 254 nm dose was measured by a sensor in the UVC light box. All cells were washed with phosphate-buffered saline (PBS), drained in a dish, and exposed to UVC radiation at a dose-rate of 0.5 J/m^2 of UVC on ice, and the comet assay was performed immediately. As for the H_2O_2 treatment, the indicated doses of H_2O_2 were added directly into the medium and cells were exposed to the indicated time.

Comet assay. The standard comet assay without enzyme digestion is described as follows: Agarose gels were made using PBS. A glass

microscope slide was placed on a hot plate at 60℃, and 100 μl of 1% normal-melting point agarose at 65℃ was applied to cover an area of 6.0 × 2.5 cm. A coverslip was applied immediately and the slide placed on ice to set the gel. Coverslips were then removed by dipping slides into water containing ice. To each gel surface, a second layer of agarose containing cells in a total volume of 80 μl was applied. This second layer was prepared by mixing 400 μl of 1.5% low-melting point agarose at 40 ℃ with 200 μl of PBS containing earthworm cells (10^3 cells/μl). Cover slips were applied immediately and slides were placed on ice to set the gel of second layer. Then the coverslips were removed again for the third layer. In a similar manner as the first layer, a 100 μl of 1.5% low-melting-point agarose was applied as the third layer. After removing coverslips, slides were immersed in 4℃ cell lysis solution for 18 h or more time as indicated. Cell lysis solution contained 2.5 M NaCl, 100 mM EDTA and 10 mM Tris; the pH was adjusted to 10.0 with NaOH and 1% *N*-laurylsarcosine, 1% Triton X-100, and 10% dimethylsulfoxide added immediately before use. DNA was then denatured by incubating in 0.3 M NaOH, 1 mM EDTA (pH 13.4) for 20 min. Electrophoresis was performed at 25 V, 300 mA for 25 min.

Slides were washed briefly in distilled water, blotted, and transferred to 0.4 M Tris-HCl, pH 7.5. DNA was stained by adding 12.5 μl of 200x Sybr green I to slides. A coverslip was applied and comets were observed under a fluorescence microscope (wavelength 450-490, farb teiler (FT) 510, long pass filter (LP) 520). The image of 50 cells per treatment was recorded with a digital camera (Kodak DCS-420). The migration of DNA from the nucleus of each cell was measured with a computer program using the parameter of comet moment. The comet moment was calculated using the formula $\sum 0 \rightarrow n$ [(amount of DNA at distance X) × (distance X)]/total DNA. This protocol is referred to as the standard comet assay.

For enzyme digestion, as performed in previous work (11, 12), after lysis treatment, slides were washed with distilled water then incubated at 37℃ for 30 min in enzyme reaction buffer [in the experiments, Trevigen; 10 mM HEPES-KOH (pH 7.4), 100 mM KCl; 10 mM EDTA, 0.1 mg/ml bovine serum albumin as Fpg digestion buffer; 10 mM HEPES-KOH (pH 7.4), 100 mM KCl, and 10 mM EDTA as endonuclease III digestion buffer]. Then 0.5 unit of UV endonuclease V (for UVC-induced CPDs), or 1 unit of Fpg followed by 1 unit of endonuclease III (for H_2O_2-induced oxidative adducts) in 10 μl of enzyme reaction buffer was added to the

center of each half of the gel. A coverslip was applied and the slides were incubated at 37℃ for 2 h in a sealed box containing wet tissue paper. Slides were prepared for electrophoresis as described above.

The enzyme-incorporated earthworm comet assay system was established, using UV endonuclease V to identify UV-induced CPD (13), and Fpg plus endonuclease III to identify H_2O_2-induced oxidative DNA adducts (14, 15).

Efficiency of earthworm testis cells at excising UVC- and H2O2-induced DNA adducts. A: Cells were pretreated with 5 mM hydroxyurea plus 50 μM cytosine-β-D-arabinofuranoside (H/A) for 0.5 h, then irradiated with 6 J/m^2 UVC, or co-treated with H/A and 80 μM H_2O_2 for 0.5 h, and then re-incubated for 0-6 h in H/A before slide making and comet assay.

Efficiency of earthworm testis cells at rejoining UVC- and H2O2-induced DNA strand breaks after cellular excision step. A: Cells were pretreated with H/A for 0.5 h, then irradiated with 6 J/m^2 UVC, or co-treated with H/A and 80 μM H_2O_2 for 0.5 h, and then re-incubated for 6 h in H/A to allow the excision step. H/A was then removed and the cells were

allowed to rejoin the DNA strand breaks for the indicated time (0-6 h) before slide making and comet assay.

Statistical analysis. Data are expressed as the mean ± SEM and analysis of variance (ANOVA) was employed for statistical analysis. Student's t-test was used in two-sample comparisons. $P<0.05$ was considered to be statistically significant.

Results

UV endonuclease V revealed that earthworm testis cells are defective in removing UV-induced DNA adducts. Earthworm testis cells were irradiated with 0, 2, 4 and 6 J/m^2 of UV on ice, and then the DNA strand breaks with UV endonuclease V digestion or sham-digestion were detected. There was a linear correlation of the comet moment and the UV dosage only after the digestion of UV endonuclease V (Figure 1A). H_2O_2-induced DNA adducts were also detected after the sequential digestion with Fpg and endonuclease III (Figure 1C). A linear correlation of the comet moment and the H_2O_2 was also found only after digestion of Fpg and endonuclease III. 6 J/m^2 caused the same amount of DNA

damage as 80 μM H_2O_2 after enzyme digestion (Figure 1A and 1C). The obvious difference between UV endonuclease V digestion and non-digestion of UV-treated cells revealed that there was still a significant quantity of UV endonuclease V-digestible adducts, i.e. CPDs, in the genome of earthworm cells irradiated with 6 J/m^2 UV (Figure 1B). In contrast, the DNA damage induced by 80 μM H_2O_2 was removed efficiently within 2 h (Figure 1D).

Earthworms are less efficient at excising UV-induced adducts than H_2O_2-induced adducts. Previously, none of the literature has investigated or reported defective steps of DNA repair in earthworm testis cells. In this study, the combined treatment with H/A was performed so as to totally block the DNA strand break rejoining step to allow investigations of excision dynamics. Optimally, H/A treatment for 6 h was able to block DNA rejoining capacity and DNA strand breaks excised from 6 J/m^2 UV-induced CPDs accumulated to the same level as those harvested immediately after UV irradiation and digested with UV endonuclease V (Figures 2A and 1A). The same H/A treatment resulted in accumulation of 80 μM H_2O_2-induced DNA strand breaks within 4 h almost to 100% of

the level shown in Figure 1C (Figures 2A and 2B). These results showed that earthworm cells identified and excised H_2O_2-induced oxidative DNA adducts significantly more efficiently than those of UV-induced CPD.

Earthworms are inefficient at rejoining UV-induced strand breaks. After treating earthworm testis cells with 6 J/m^2 of UVC or 80 μM H_2O_2, resulting in a similar level of strand breaks excised by H/A for 6 h, the H/A-containing medium was replaced with fresh PBS for 6 h, allowing the cells to rejoin any strand breaks. H_2O_2-induced DNA strand breaks were rejoined to the basal level within 2 h, indicating that the DNA strand break rejoining capacity for H_2O_2 induced damage was significantly efficient (Figures 3A and 3B). The removal of H/A-containing medium from cells irradiated with 6 J/m^2 of UVC and treated with H/A led only to rejoining 65% of the DNA strand breaks after 6 h (Figures 3A and 3B). The obviously different patterns in rejoining dynamics for UVC and H_2O_2 showed that earthworm testis cells were capable of rejoining UV-induced DNA strand breaks but not as efficiently as for these induced by H_2O_2 for those of UVC.

Discussion

UV is known to induce CPDs and photoproducts, the former being much more important in mammalian cells (16). UV-induced CPDs are mended by the NER pathway (7). It has been reported that H_2O_2 treatment produces large quantities of 8-hydroxyguanine in cultured mammalian cells (17, 18), together with other adducts, such as thymine glycol (19, 20) and imidazole ring-opened derivative of guanine designated 2,6-diamino-4-hydroxy-5-24 formamidopyrimidine (17). H_2O_2-induced DNA adducts were diverse (15, 16, 20) and are repaired by the base excision repair (BER) pathway. In this study, we used an enzyme-incorporated comet assay to detect dynamic removal of oxidative adducts induced by UVC and H_2O_2 from the earthworm genome with the digestion of UV endonuclease V and Fpg plus endonuclease III. Fpg protein can cleave oxidative bases such as 8-oxoguanine, 5-hydroxycytosine, 5-hydroxyuracil, 2,6-diamino-4-hydroxy-5-*N*-methylformamidopyrimidine, and 4,6-diamino-5-formamidopyrimidne (14). Endonuclease III can cleave many pyrimidine derivatives, including thymine glycol, 5,6-dihydrothymine, 5-hydroxy dihydrothymine, 5-hydroxycytosine, 5-hydroxyuracil, and uracil glycol (15). H/A blocking of the rejoining

step was also adopted to further identify the functions of DNA repair steps of earthworm cells in accordance with our previous work [19, 20]. The results showed that earthworm testis cells were possibly not only efficient in the excision step (Figure 2), but also for the rejoining step of the BER (Figure 3). Noticeably, earthworms were defective in both the excision and rejoining steps of NER (Figures 2 and 3). The detailed mechanisms of the DNA repair proteins involved need further investigation.

In addition to being a pilot study of DNA repair capacity in earthworms, this paper has provided molecular evidence for DNA repair of earthworms. Firstly, the defects in both the excision and rejoining pathways for repair of UV-induced CPDs may explain why earthworms avoid sunlight. Secondly, the high efficiency in removing the Fpg- and endonuclease III-digestible adducts may provide another explanation, in addition to their higher antioxidant capacity, for their high resistant to pollutant heavy metal toxicity, which may cause high oxidative stress to both the environmental soils and living creatures.

There were some limitations in this pilot study and some promising directions for us to explore in the future. Firstly, UV did not penetrate the

skins and directly irradiate testis cells. It would also be very meaningful to compare the effects of UV on skin cells of earthworms with those of humans. Secondly, UV used here was UVC and it would be interesting to compare the effects of UVA and UVB, in addition to UVC on earthworm skin cells. Normally, the stratospheric ozone reflects UVC and most of the UVB, so only UVA and a little UVB reach the earth (21). However, the ongoing atmospheric ozone depletion has strengthened the importance of studies of UVA- and UVB-induced DNA damage and their effects on the earth's creatures at cellular and physiological levels. It has been reported that a 10% decrease in total stratospheric ozone would increase the amount of UVB reaching the earth's surface by 20% (22), and some models of UVB on earthworms have already been developed (9, 10), showing that earthworm skin and muscle cells are good models for investigating photodamage. However, our study is the first one to study the UVC-induced effects on the testis cells of earthworms.

Conclusion

Our findings provide evidence for the retardation of UV-induced CPD excision and defects in DNA strand break rejoining in earthworms, and

may explain why earthworms avoid constant sun exposure.

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Figure Legends

Figure 1. The overall UVC-induced and H_2O_2-induced DNA repair dynamics in earthworm testis cells. A: Cells were irradiated with indicated dosages of UVC (0-6 J/m^2) on ice. Then the cells are subjected to slide making immediately after the UVC irradiation. The slides were sham-digested (△) or digested with UV endonuclease V twice (▲), and then assessed for DNA strand breaks by comet assay. *P<0.05, with *versus* without UVC irradiation (UV=0); P<0.05, with *versus* without UV endonuclease V digestion. B: Cells were irradiated with 6 J/m^2 UVC and re-incubated for the indicated time (0-6 h) before slide preparation. Then the slides were sham-digested (△) or digested with UV endonuclease V for twice (▲), and then DNA strand breaks were detected by comet assay. □ represented cells without UVC irradiation but with UV endonuclease V digestion. *P<0.05, with *versus* without repair (0 h); P<0.05, with *versus* without UV endonuclease V digestion. C: Cells were treated with the indicated dosages of H_2O_2 (0-80 μM) for 0.5 h on ice. Then the cells were subjected to slide making immediately. The slides were sham-digested (▽) or digested with Fpg and endonuclease III (▼), and then examined for their DNA strand breaks by comet assay. *P<0.05,

with *versus* without H_2O_2 treatment; P <0.05, with *versus* without Fpg and endonuclease III digestion. D: Cells were treated with 80 μM of H_2O_2 and re-incubated for the indicated time (0-6 h) before slide preparation. Then the slides were sham-digested (▽) or digested with Fpg and endonuclease III (▼), and then DNA strand breaks were detected by comet assay. □ represented cells without H_2O_2 treatment but with endonuclease V digestion. *P<0.05, with versus without H_2O_2 treatment; P<0.05, with *versus* without Fpg and endonuclease III digestion.

Figure 2. Efficiency of earthworm testis cells at excising UVC- and H_2O_2-induced DNA adducts. A: Cells were pretreated with 5 mM hydroxyurea plus 50 μM cytosine-β-D-arabinofuranoside (H/A) for 0.5 h, then irradiated with 6 J/m^2 UVC (△), or co-treated with H/A and 80 μM H_2O_2 for 0.5 h (▽), and then re-incubated for 0-6 h in H/A before slide making and comet assay. *P<0.05, *versus* no repair time (R0); P<0.05, UVC *versus* H_2O_2. B: The excision efficiency for each time point was calculated by the formula: point excision efficiency=point excision comet moment (Rx-R0)/maximum excision comet moment (R6-R0) × 100%; maximum excision comet moments were 51.87 for UVC and 52.49 for

H_2O_2 groups, respectively.

Figure 3. Efficiency of earthworm testis cells at rejoining UVC- and H_2O_2-induced DNA strand breaks after cellular excision step. A: Cells were pretreated with 5 mM hydroxyurea plus 50 μM cytosine-β-D-arabinofuranoside (H/A) for 0.5 h, then irradiated with 6 J/m^2 UVC (△), or co-treated with H/A and 80 μM H_2O_2 for 0.5 h (▽), and then re-incubated for 6 h in H/A to allow the excision step. H/A was then removed and the cells were allowed to rejoin the DNA strand breaks for the indicated time (0-6 h) before slide making and comet assay. *P<0.05, *versus* no repair time (R0); P<0.05, UVC *versus* H_2O_2. B: The rejoining efficiency for each time point was calculated by the formula: point rejoining efficiency=point rejoining comet moment (R0-Rx)/maximum rejoining comet moment (R0-R6) × 100%; maximum rejoining comet moments were 52.13 for UVC and 52.37 for H_2O_2 groups, respectively.

Figure 1

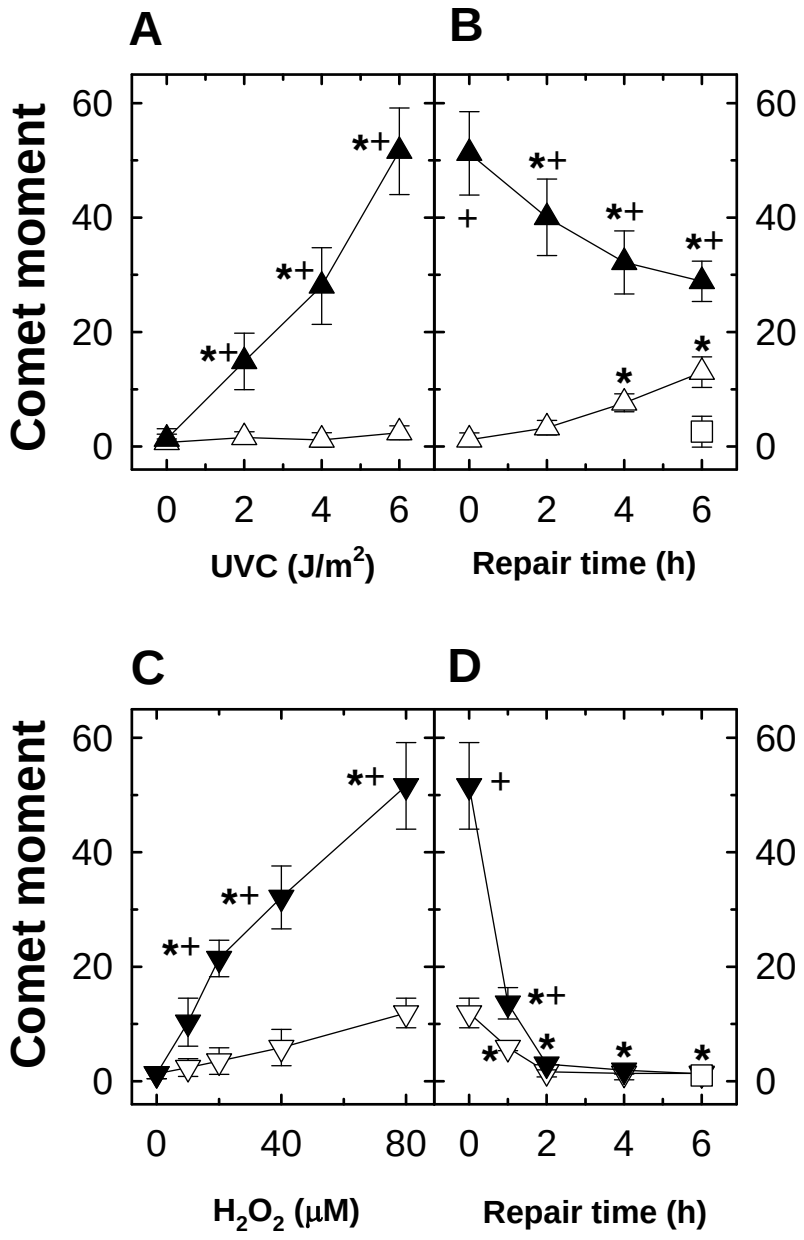
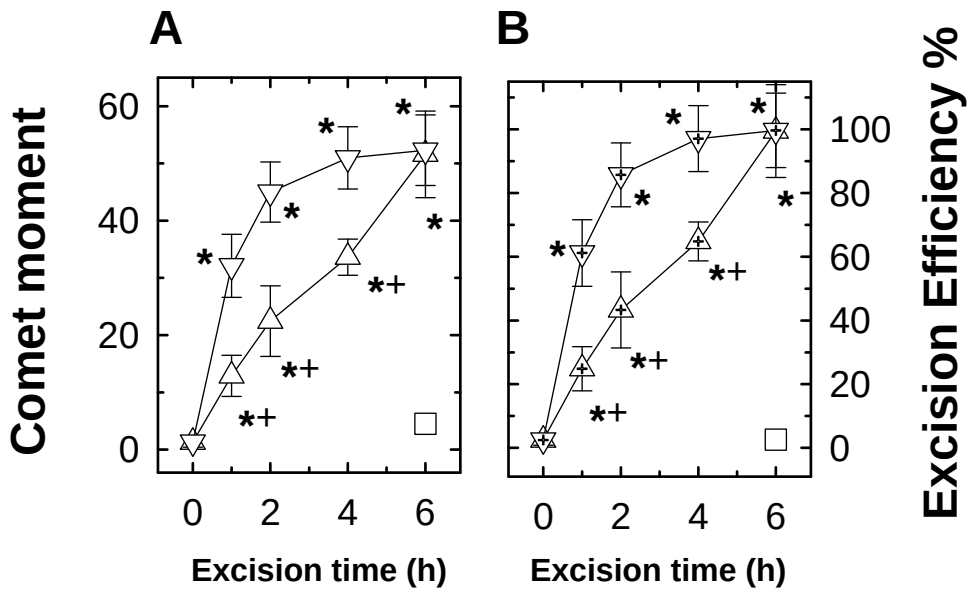


Figure 2


A
B
Comet moment
Excision Efficiency %
Excision time (h)
Excision time (h)
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20
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2
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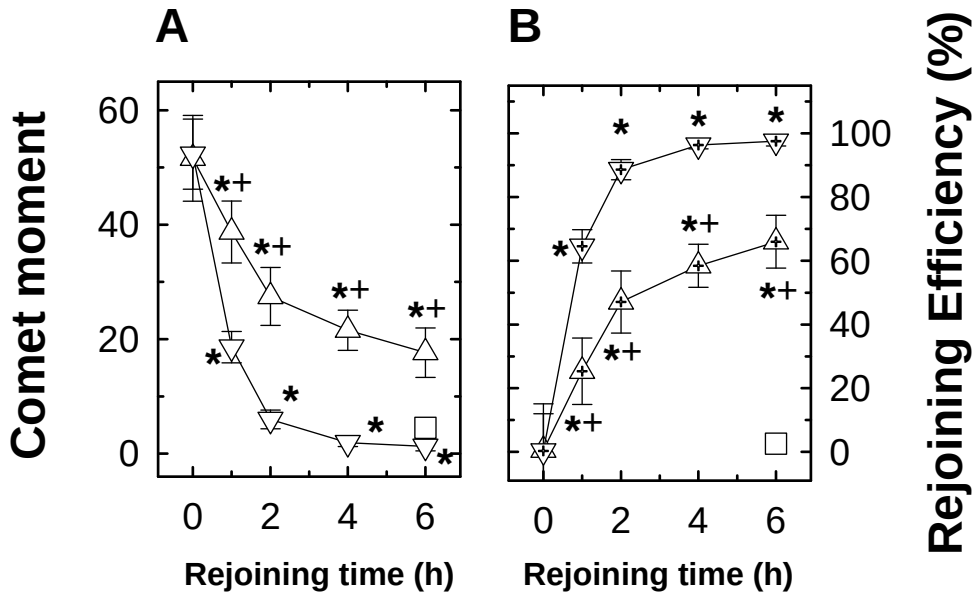
6

Figure 3


A
B
Comet moment
Rejoining Efficiency (%)
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20
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Rejoining time (h)
Rejoining time (h)