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Mutagenic Effects of Potassium Dichromate as Evaluated by Means of Animal and Plant Bioindicators

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Abstract. *Background: Chromium typically occurs in two oxidation states in the natural environment, Cr^{3+} [Cr(III)] and Cr^{6+} [Cr(VI)]. Out of the two chromium species, Cr(VI) is the most mobile, labile and toxic. Hexavalent chromium [Cr(VI)] compounds are classified by the International Agency for Research on Cancer (IARC) as carcinogenic agents to humans. The main source of release of chromium in aquatic ecosystems is related to the industrial application of this metal in metallurgies, tanneries, and in the manufacturing of paints and dyes. The ecotoxicology of Cr(VI) is linked to its environmental persistence and the ability to induce adverse effects in biological systems. In the present study, we evaluated mutagenic effects of Cr(VI) in animal and plant bioindicators. Materials and Methods: We evaluated primary DNA damage and frequencies of micronuclei (MN) and morphological nuclear abnormalities (NA) in erythrocytes in peripheral blood of the fish Oreochromis niloticus exposed to potassium dichromate at 12 mg l^{-1}. The genotoxicity and cytotoxicity of Cr(VI) in the onion (Allium cepa) test were also assessed. Results: The comet assay showed a significant increase of tailed nucleoids in the erythrocytes of fish treated with $K_2Cr_2O_7$; MN frequency was also increased in the treatments; cytotoxicity of a low concentration of potassium dichromate, however, was not confirmed. Conclusion: The combination of both systems – animal and plant – is adequate and advantageous for mutagenicity evaluation. The findings indicate that at the concentration tested, the chromium compound is a clastogenic as well as an aneugenic.*

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In the environment, chromium exists in two main oxidation states, Cr^{3+} [Cr(III)] and Cr^{6+} [Cr(VI)], that have different geochemical and biological activities, since Cr(III) is an essential metal nutrient and Cr(VI) is carcinogenic (hexavalent chromium compounds are classified by the International Agency for Research on Cancer (IARC) as group 1 agents, *i.e.* carcinogenic to humans). Although Cr(VI) is more mobile, labile and toxic than Cr(III), the distribution of both is regulated by redox reactions in natural waters. Under acidic and reducing conditions, Cr(III) species will predominate in water, while Cr(VI) species will prevail under alkaline and mildly oxidizing conditions (1).

A great source of Cr(VI) is potassium and sodium dichromate, that are used in the chrome industry, in manufacturing pigments and dyes, in tanneries, in the preparation of antiseptics, in cleaning glassware laboratory and as a titrant, among others (2). Thus, the main source of release of chromium into aquatic ecosystems is related to the industrial application of this metal (2-4).

Incidents of groundwater pollution with chromate have occurred in many sites. One such occurrence took place in Long Island, New York, in which chromate moved from an industrial waste disposal pit to contaminate shallow groundwater. It persisted to a concentration as great as 12-16 mg l^{-1} of Cr(VI) more than 900 m away from the original source. It was not until 20 years later that the contamination was first noticed in water (5). This event is an example of the importance of studying and understanding how this type of pollution affects the environment.

Analyses on potassium dichromate mutagenicity have already been conducted in aquatic organisms such the algae *Pseudokirchneriella subcapitata* (6), the crayfish *Procambarus clarkii* (7) and fish species (2, 8-12).

Fish are particularly sensitive to environmental contamination of water since pollutants may significantly interfere with several of their biochemical processes (13). Fish have been successfully used in cytogenetic analysis as they are easy to handle and keep in the laboratory, besides

providing a relatively low-cost method (14). The use of fish erythrocytes allows for quick results with little suffering on the part of the organisms used in bioassay monitoring (15).

The comet assay is a fast, reliable, sensitive and cost-effective environmental tool for the assessment of genotoxic insult (16). This assay is usually performed with blood cells due to their easy access and also the composition of 97% nucleated erythrocytes (17).

When compared to other techniques for DNA damage detection, the micronucleus (MN) assay has some advantages: (i) it can be performed rapidly; (ii) it is not complex; (iii) it presents low costs; (iv) its preparation and analysis are simpler and faster than studying chromosomal aberrations. Although the MN assay cannot give information about the type of chromosomal breakage, it is informative when the exposure causes aneugenic effects. Taken together, all these aspects render this methodology high applicable in routine mutagenesis studies (18). For quite some time, considerable attention has been paid to the simultaneous presence of morphological nuclear abnormalities (NA) and MN in the piscine MN test (19).

In addition, the bioindicative potential of low trophic-level organisms has increasingly been investigated to assess the possible environmental hazards associated with contaminant transfer along different food webs (20), that justifies the use of producers as bioindicators.

In bioassays with the onion, *Allium cepa*, after specific exposure to a compound, it is possible to evaluate cytotoxic effects manifested as a reduction in root growth or a decrease in the mitotic index, as well as genotoxic effects, usually by analysis of MN or abnormalities in cellular division (21). The *Allium* root chromosomal aberration assay was validated in 1991 by the International Programme on Chemical Safety under the auspices of the World Health Organization, and the United Nations Environment Programme. This bioassay has proven to be an efficient test for chemical screening, especially for monitoring the genotoxicity of environmental pollutants (22).

Aiming to address the mutagenic effects of hexavalent chromium in both animal and plant bioindicators, the study described here used the erythrocyte comet assay and MN test in the fish *Oreochromis niloticus* and the cellular cycle of *Allium cepa* root meristem cells.

Materials and Methods

Experimental design. Potassium dichromate ($K_2Cr_2O_7$, CAS Registry Numbers: 7778-50-9, M.W. 294.18) was tested for its genotoxic and cytotoxic properties in the present study.

Oreochromis niloticus (Linnaeus, 1757), commonly known as tilapia, was chosen as a model because it is easy to adapt and maintain under laboratory conditions. Specimens of *O. niloticus*, with an average length of 16.79±1.78 cm and an average weight of 83.76±19.55 g, were obtained from the Pesque & Pague Company (Ananindeua, Pará State, Brazil). Prior to the experiment, fish were acclimated to experimental conditions for 30 days (one fish per each 30-l aquarium in dechlorinated tap water, with constant aeration, temperature 26±1.3˚C, 12 h:12 h light:day photoperiod) and one-third of the total water volume was changed every 48 h. Seven specimens were first used as a negative control and then exposed to 12 mg l^{-1} potassium dichromate (concentration obtained by adding 360 mg of $Cr_2K_2O_7$ in each aquarium). This concentration was based on that occurring in contaminated water at Long Island reported by Perlmutter *et al*. (5), and on that of Normann *et al*. (8) who evaluated the potassium dichromate impact on the armored catfish *Hypostomus plecotomus* through MN testing. Before exposure (for the negative control) and after 24- and 48-h exposures, blood samples (0.5 to 1 ml) were collected from the tail vein for the comet assay and MN test.

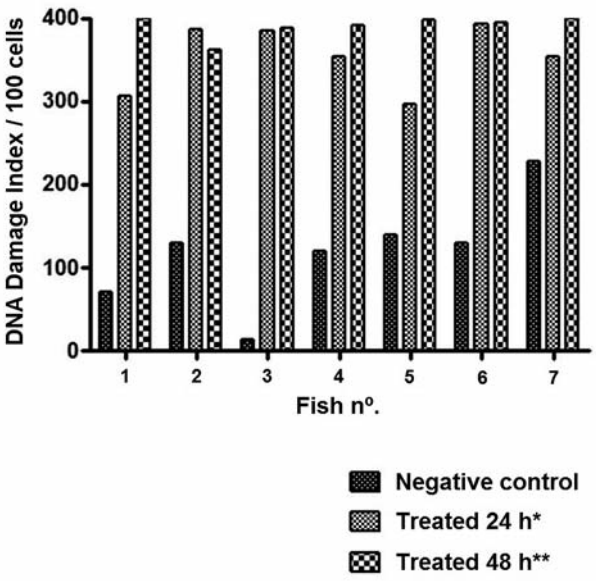

Figure 1. *Damage index (DI) by the Comet assay (score of genetic damage) for blood cells evaluated in Oreochromis niloticus after exposure to potassium dichromate. *p<0.05 and **p<0.005 compared to negative control.*

A. cepa was used as a bioindicator plant and in this case the biomarkers were mitotic index, chromosomal aberrations, and MN evaluated in the root meristem obtained from germination of seeds exposed to 12 mg l^{-1} potassium dichromate.

Comet assay and MN test using O. niloticus. Blood samples were obtained from seven specimens of *O. niloticus* by tail puncture using heparinized syringes. The comet assay was performed as described by Östling and Johansson (23), with minor modifications (24). Two slides were prepared from each specimen for each exposure time. Initially, an aliquot of blood (10 μl) was diluted in 1 ml of fetal calf serum. Subsequently, 10 μl of this cell suspension was dissolved in 120 μl of 0.75% low melting point agarose and immediately spread onto a glass microscope slide pre-coated with a layer of 1% normal melting point agarose.

The agarose was allowed to set at 4˚C for 5 min. The slides were then incubated in ice-cold lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% Triton X-100 and 10% DMSO, pH 10.0) at 4˚C

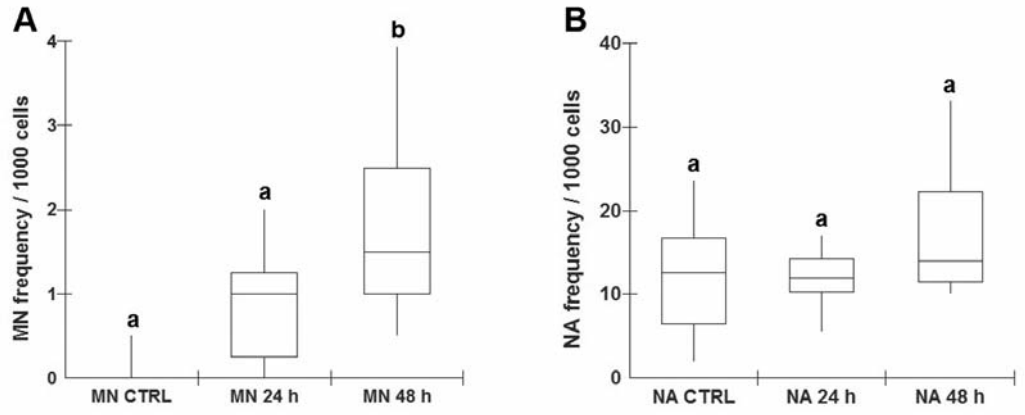

Figure 2. *Piscine MN test of blood cells from Oreochromis niloticus exposed to potassium dichromate. MN frequencies (A) and NA frequencies (B). The line in boxes corresponds to the median value, and the lower and upper boundaries of the boxes to the first and third quartiles, respectively. The lowest and highest scores are indicated by bars. Groups not sharing the same letter are significantly different at p<0.05 by Kruskal–Wallis ANOVA followed by SNK test. CTRL: Control.*

for at least 1 h to remove cellular proteins, leaving the DNA as 'nucleoids'. After the lysis procedure, the slides were placed on a horizontal electrophoresis unit. The unit was filled with fresh buffer (300 mM NaOH and 1 mM EDTA, pH>13.0) to cover the slides for 20 min at 4˚C to allow DNA unwinding and expression of alkali-labile sites. Electrophoresis was then conducted for 20 min at 25 V (300 mA). All the above steps were conducted under yellow light or in the dark to prevent additional DNA damage. The slides were then neutralized (0.4 M Tris, pH 7.5), dried with absolute ethanol, stained with ethidium bromide (20 µg/ml), and analyzed using a fluorescence microscope, at a magnification of ×400.

One hundred randomly selected cells (50 cells from each slide) were analyzed. Cells were scored visually according to tail length into five classes: class 0: undamaged, without a tail; class 1: with a tail shorter than the diameter of the head (nucleus); class 2: with a tail length 1-2 times the diameter of the head; class 3: with a tail longer than twice the diameter of the head; class 4: comets with no heads. The damage index (DI) was assigned to each comet according to its class, using the formula:

$DI=(0 \times n_0) + (1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4)$,

where n=number of cells in each class analyzed. The DI ranged from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4) (25, 26).

For the MN test, blood smears were prepared on slides, with 14 slides being prepared (two from each fish) for each exposure time. The material was fixed in absolute ethanol for 10 min and stained with 5% Giemsa for 20 min. The number of normal erythrocytes without MN and the number of damaged cells with MN or morphological NA (blebbed, notched and lobed nuclei) were determined by analysis of 2000 cells per fish (27). Among current cytogenetic techniques, NA and MN are considered as indicators of cytotoxicity and genetic toxicology, respectively (28).

Onion root-tip genotoxicity and cytotoxicity tests. For this test onion (*A. cepa* variety Periform Baia, provided by Feltrin Ltd., of Santa Maria, RS, Brazil) seeds were germinated in Petri plates. On the fifth day (after 120 h) the radicles had reached 1.5 to 2 cm in length. The treatments were: T0, sterile distilled water only (negative control); T1, 96 h with distilled water and 24 h with a 12 mg l^{-1} solution of potassium dichromate; and T2, 72 h with distilled water and 48 h with a 12 mg l^{-1} solution of potassium dichromate.

After treatment, the roots were fixed in 3:1 (v/v) methanol/glacial acetic acid (Carnoy solution) for 24 h. They were then carefully squashed and hydrolyzed with 1 N HCl for 15 min, washed with distilled water and stained with 2% acetic orcein for 10 min. The analysis of mitotic index was carried out on 5000 cells per treatment. All cells with alterations were counted and the most representative ones for each abnormality were photographed. For the mutagenicity assessment, besides MN (interphase anomalies), mitotic anomalies (irregular metaphase, anaphasic bridge, telophasic bridge, multipolar mitosis, and cells with chromosomal loss) were also recorded.

Statistical analysis. Data were tested for normality of distribution and homogeneity of variances. The Lilliefors test revealed significant departure from normality and Cochran test demonstrated heteroscedasticity. Kruskal–Wallis non-parametric ANOVA was applied for detecting differences at the 0.05 significance level. Differences between ranks were compared by means of Student-Newman-Keuls (SNK) method. Analyses were undertaken with the statistical package BioEstat 5.0 (29) and GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA).

Results

The *O. niloticus* comet assay and the MN test showed that the potassium dichromate at 12 mg l^{-1} was significantly genotoxic and mutagenic compared to the water used on the negative control. However, in the present experiment, the number of damaged erythrocytes with NA in the exposed groups was not statistically different compared to the control group (Figures 1 and 2).

In the onion root-tip, mutagenicity tests were significantly different. A significant increase in the frequency of MN was

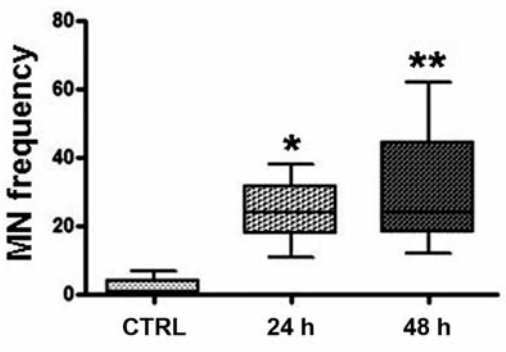

Figure 3. *MN frequency (measurement of genotoxicity) evaluated in the bioassay of meristematic cells of Allium cepa exposed to potassium dichromate. The line in boxes corresponds to the median value, and the lower and upper boundaries of the boxes to the first and third quartiles, respectively. The lowest and highest scores are indicated by the bars. Significantly different from the control (CTRL) at *p<0.05 and **p<0.01 (Kruskal–Wallis ANOVA, followed by SNK test).*

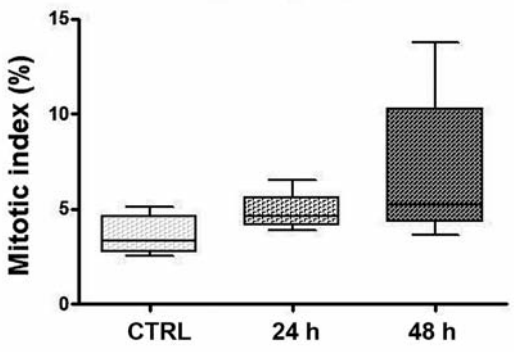

Figure 4. *Mitotic index (measurement of cytotoxicity) evaluated in the bioassay of meristematic cells of Allium cepa exposed to potassium dichromate. The line in boxes corresponds to the median value, and the lower and upper boundaries of the boxes to the first and third quartiles, respectively. The lowest and highest scores are indicated by the bars. There was no significant difference between groups (p=0.0750, Kruskal–Wallis).*

observed in treated groups (Figure 3) (Kruskal-Wallis, p=0.0082). Furthermore, mitotic anomalies were observed only in groups exposed to potassium dichromate. However, in the onion root-tip cytotoxicity test, the two exposure times to potassium dichromate did not lead to a significant difference from the data of the control group in regards to the mitotic index (Figure 4).

Discussion

Hexavalent chromium compounds are classified by the International Agency for Research on Cancer as being carcinogenic to humans. Cr(VI)-induced DNA–protein crosslinks have been implicated in the mutagenic and carcinogenic effects of Cr(VI). Cr(VI) exposure increases the cellular level of protein carbonyls and Cr(VI)-induced DNA-protein crosslinks may be formed, at least in part, *via* the generation of such carbonyls (30).

The assessment of DNA damage when using the comet assay arises from the complex interaction between two processes: DNA damage and its repair (activation or inhibition). The damage in this case indicates a recent response to a pollutant (genotoxic damage). On the other hand, when MN are formed, they remain present throughout the lifetime of the cell (31). The combined approaches using both these assays, comet and MN, is adequate and advantageous, and will help in providing a broad perspective in aquatic toxicology (12, 32). The level of damage detected by the comet assay is usually higher than that found in the MN test. In some cases, primary damage is often not inherited by future cellular generations and, for this reason, cannot be detected by the MN assay (32)

The higher plants, *A. cepa* (onion), *Tradescantia paludosa* and *Vicia faba*, have relatively large monocentric chromosomes in reduced numbers and are accepted as suitable test organisms for the study of environmental mutagenesis (33, 34). A number of authors suggest that different plant test systems are useful for studying the cytotoxicity and genotoxicity of heavy metals (22, 34-36).

Genotoxicity. The comet assay showed a significant increase in the frequency of tailed nucleoids in treated fish, thus indicating hexavalent chromium-induced DNA damage. Our results agree with those reported by Blasiak and Kowalik (37) and Matsumoto *et al.* (38), who proposed that chromium exerts a genotoxic effect on animals due to its potential to cause various forms of DNA damage.

Arunachalam *et al.* used the comet assay in the evaluation of genotoxicity of hexavalent chromium in fingerlings of *Catla catla*, a major Indian carp (11). There was a general increase in the DNA damage values with increasing concentration of chromium and exposure time. In our study, more significant damage was also observed during longer exposure. In addition to observing that Cr(VI) promoted a concentration- and time-dependent DNA damage in *Channa punctatus,* Kumar *et al.* also found that gill cells showed comparatively higher DNA damage compared to blood cells at all test concentrations and sampling times (10).

While spontaneous (or basal) MN frequency in fish is normally very low (39), appreciable inter-species differences have been reported. Thus, per 1,000 cells, the average MN frequency was 0.08±0.13 in *Hoplias malabaricus* (39), 0.1±0.316 in *Eigenmannia virescens* (40), 2.4±1.19 in

Colossoma macropomum (41), and 0.07±0.188 in *O. niloticus* (present study). Furthermore, in MN assays undertaken by Ramsdorf *et al*. using *H. malabaricus* there were no MN, only morphological NA (42).

In the present work, a statistically significant increase ($p<0.05$) in MN in erythrocytes was indeed observed after exposure of fish to Cr(VI) for 48 h. The mean increased to 1.86±1.345 per 1,000 cells, *i.e*., an increase of more than 25-fold relative to the basal MN frequency. This result suggests that *O. niloticus* is more sensitive to chromium than is the armored catfish *Hypostomus plecotomus* used in the experiment of Normann *et al*. in which exposure to chromium for 15 days led to increased MN frequency 11-fold higher than the basal level (8). In a recent publication, our group reported a significant increase in MN and NA frequencies in tilapia after 48 h exposure to Cr(IV) (9).

The mutagenicity of Cr(VI) was also demonstrated in meristematic onion cells, by the appearance of mitotic abnormalities occurring in treated groups. The most frequent abnormalities were cells with chromosomal loss and irregular metaphase. In addition, there was a significant increase in the frequency of MN in cells exposed to chromium, some of which exhibited up to three MN.

Some onion cells exhibited the loss of fragments or whole chromosomes, that persisted up to telophase, such losses probably resulting in MN. These results agree with those obtained by Dash *et al*. (43) and Matsumoto *et al*. (38), who reported MN induction involving the mitotic spindle and consequent production of laggard chromosomes during anaphase and the loss of a complete chromosome.

Cytotoxicity. In the present study, no significant difference was found in the mitotic index in treated cells of *Allium cepa* roots. However, there are many studies in which chromium compounds seem to be cytotoxic, leading to a decrease in mitotic index in different higher plants. For example, Srivastava and Jain investigated the cytotoxic effects of graded concentrations of Cr(VI) on a sugarcane cultivar CoLk 8102 (hybrid *Saccharum* species) through a root-tip assay at an early stage of growth (44). The mitotic index decreased with increasing concentration of chromium. A decrease in mitotic index was observed at all concentrations of chromium, thereby indicating a mitodepressive effect of chromium treatment on cell division activities in root-tip cells.

Liu *et al*. studied the effects of trivalent and hexavalent chromium on root growth, cell division, and chromosome morphology of *A. cepa*. Onion roots were exposed to chromium nitrate and potassium dichromate (45). The mitotic index decreased progressively with increasing chromium concentration and duration of treatment. Furthermore, the mitotic index was somewhat higher in the groups treated with Cr(III) than with Cr(VI).

In fact, the cytotoxic potential of chromium compounds at low concentrations has not been confirmed. Accordingly, Mishra *et al*. tested genotoxicity and cytotoxicity of chromium trioxide (10, 20, 30, 40 and 50 mg l^{-1}) in a study using the *Vicia* bioassay (46). The data were statistically analyzed using ANOVA and significant differences between the means were assessed by Dunnet multiple comparisons test. The mitotic indices in the root tip cells of chromium trioxide-treated roots were significantly lower than that of the control. However, when we analyzed only the results for the concentration of 10 mg l^{-1} of this chemical, there were no significant differences compared to the negative control. In another study by Zou *et al*. (47), seeds of *Amaranthus viridis* were exposed to potassium dichromate solutions (10^{-6} M to 10^{-3} M) for 24, 48 and 72 h. The mitotic index decreased progressively with increasing Cr(VI) concentration, except for the seedlings exposed to 10^{-5} M Cr(VI). At 10^{-6} M Cr(VI), the mitotic index was slightly higher than that of the control 24 h after treatment, but lower than the control when increasing the duration of time. At 10^{-5} M Cr(VI), the mitotic index was higher than that of the control during the entire experiment. At 10^{-3} M Cr(VI), the mitotic index was extremely low because there were no dividing cells after treating for 72 h. These two results are interesting because the concentration of $K_2Cr_2O_7$ in our experiment was 12 mg l^{-1}, which corresponds to approximately 4.08×10^{-5} M.

A new contribution to the subject is the recent publication of Hou *et al*. (48). The researchers aimed to determine the toxic effects of chromium on seed germination of various plants and identify sensitive plant species and appropriate bioassays for potential use in phytotoxicity assessment of chromium in soil. One of the most interesting findings was that lettuce significantly accumulated chromium at all tested concentrations. However, corn and wheat significantly accumulated chromium only at the highest tested dose and this may explain the greater inhibitory effects of chromium on root growth in lettuce. These results are valuable for understanding the toxic effect of chromium on crops. Therefore, the difference in sensitivity of the test organism may also influence the cytotoxic effects tolerated.

Conclusion

O. niloticus proved to be very sensitive to the genotoxic potential of chromium, with the comet assay showing significant results in DNA damage associated with the treatment period. Although the cytotoxicity of a low concentration of potassium dichromate has not been confirmed, the findings suggest that this chromium compound is clastogenic as well as aneugenic, as demonstrated in both animal and vegetal systems.

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