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Comparative Cr, As and CCA induced cytotostaticity in mice kidney: a contribution to assess CCA toxicity

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Highlights

- CCA has toxic effects on the cell cycle dynamics of mouse kidneys, distinct from its compounds.
- CCA and As compounds showed clastogenic effects, particularly in the S phase
- Cell cycle dynamics is a valuable endpoint to assess the putative toxicity of remaining CCA-treated infrastructures
- Genotoxicity of CCA and its constituents in renal cells claims for their ecofriendly removal prior leakage.

Abstract

CCA (Chromium Copper Arsenate) treated wood, widely used in outdoor residential structures and playgrounds, poses considerable dangers of leaching of its components to the environment.

In this study, mouse kidney samples were used to evaluate the effects of CCA, chromium trioxide (CrO_3) and arsenic pentoxide (As_2O_5) on cell pathophysiology by flow cytometry. Samples were collected after 14, 24, 48 and 96 hours of animal exposure. While Cr had no statistically significant cytostatic effects, As_2O_5 induced a S-phase delay in animals exposed for 24h, and over time a G0/G1 phase blockage. The effects of CCA in S-phase were similar, but more severe than those of As_2O_5. Since environmental and public health hazards due to the long durability of CCA-treated wood products, these data confirm that CCA has profoundly toxic effects on cell cycle, distinct from the compounds themselves. These cytostatic effects support cell cycle dynamics as a valuable endpoint to assess the toxicity of remaining CCA-treated infrastructures, and the expected increased waste stream over the coming decades.

Keywords: CCA, arsenite, chromium, clastogenicity, cytostaticity, kidney

1. Introduction

Timber additives are chemicals used worldwide in the manufacture of wood. Chromated copper arsenate (CCA), a mixture of chromium trioxide (CrO_3), copper oxide (CuO) and arsenic pentoxide (As_2O_5) is a lumber preservative used to protect wood against deterioration caused by insects and microbial agents (Barraj et al., 2009). CCA treated wood has been widely used in outdoor residential structures and public playgrounds, but US legislation (USEPA, 2002) and the European Union (Annex XVII to Regulation (EC) No 1907/2006 on the Registration, Evaluation and Authorization of Chemicals, REACH), has severely restricted its use. In China, such limitation has not yet been established and wood treated with CCA is still widely applied in public parks (Tang et al., 2015). Recently, the levels of As in the soils adjacent to CCA-wood were reported to be as high as 110 mg/kg (Gress et al., 2016), with As above USEPA ecological soil screening levels (USEPA, 2003, 2005). As has been described as more mobile than chromium being detected at deeper distances in soil under preserved wood (Zagury et al., 2008). Climatic factors and amount of applied CCA, among others, influence the leaching rate of its components. As revised by Hingston et al (2001), the leaching of the individual components is not proportional to the concentrations of the individual components in the original CCA formulation. Studies show that in more modern formulations, Cu and As are lost in highest degree, despite being present in smallest proportions in the formulation. For instance, the leaching rates for Cu and As in seawater after 21 days were described to be approximately 1 mg cm^{-2} day^{-1} and 0.1 mg cm^{-2} day^{-1} for Cr (Merkle et al., 1993). Leaching rate is strongly dependent of several factors, namely the wood block size, the leaching media pH and salinity, and the temperature. Low pH increases the leaching rates, while lower temperatures decrease the leaching rate. For instance, leaching of Cr was reported to be 0.119 µg m^{-2} s^{-1} and 0.079 µg m^{-2} s-1 at 20ºC and 8ºC, respectively (Van Eetvelde et al., 1995) Regarding the effect of the salinity, solutions of higher ionic strength show higher leaching rates of CCA (Irvine and Dahlgren, 1976). Moreover, studies by Breslin and Adler-Ivanbrook (1998) also show that the leaching rates of all components tend to decrease with time.

Due to the extensive and long-lasting use (20/50 years) of CCA-treated wood, a large increase in the residues is expected in the next decades (Choi et al., 2012; Mercer and Frostick 2012). Efforts have been made to study the impact of CCA-leachate from treated wood waste, addressing both the toxicity of CCA and its constituents (Mercer and Frostick 2012). These authors showed that As, Cr and Cu leached from CCA-wood waste occasionally appeared in the environment in concentrations that exceed regulatory limits in two to three orders of magnitude, underlining the toxicological risks of this wood waste as a pollutant, both in the environment and in human health. After compiling different toxicity studies on the effects of CCA treated wood, Katz and Salem (2005) concluded the need to clarify their chemistry and toxicological effects.

Chromium and arsenic are known to be potent carcinogens (Roy and Saha 2002; World Health Organization 2010; Ohgami et al., 2015). Their release from CCA treated residential, including staircases at apartment complexes in Florida (Gress et al., 2014) and outdoor structures, including playground structures used by children prompt many studies (Shalat et al., 2006, Zatarian et al., 2006, Barraj et al., 2009, Tang et al., 2015). Very recently Deramos and colleagues (2019) reported on the persistence of arsenic in soils surrounding old playground structures as a source of exposure for small children through dermal contact and ingestion of contaminated soil (owed to children hand-to-mouth activity).

In a study with mice as models, Mason and Edwards (1989) evaluating the nephrotoxicity of $Na_2Cr_2O_7$, $CuSO_4$ and Na_3AsO_4, showed that the mixture of these three compounds had higher risk of toxicity than their individual components. Chromium and arsenic are also known as nephrotoxic agents (Madden and Fowler 2000, Fatima et al., 2005). Pentavalent and hexavalent chromium induce the formation of cylinders and damage to renal brush border in rodents (Fowler 1993, Fatima et al., 2005, Oliveira et al., 2006a). Matos et al. (2009a) reported for ICR-CD1 mice that CCA, CrO_3 and As_2O_5 differed in their accumulation profiles in kidney. They observed histopathological differences in the kidneys of animals treated with CCA and CrO_3, including the appearance of casts and epithelium desquamations (Matos et al., 2009a). Mice treated with commercial solutions of CCA showed higher nephrotoxicity than those treated

with equivalent solutions of the individual compounds, i.e., CrO_3 and As_2O_5 (Matos et al., 2009b). More recently, Takahashi and colleagues (2018) reported deleterious effects on multiple organs of rats exposed to CCA for one month. However, little attention has been paid to the effects of CCA and its components on the genotoxicity and cell cycle dynamics of exposed organisms. In the present study, Cr, As and CCA induced cytotostaticity in mice kidney was studied to evaluate the impact of CCA on cellular pathophysiology.

2. Materials and Methods

2.1 Chemicals and Reagents

A commercial Type C CCA solution (purchased from a local wood industry containing 47.5% CrO_3, 18.5% CuO and 34.0% As_2O_5 (w/w) was used to prepare a two-hundred-fold dilution, having adjusted to pH 7.0. This first solution was analyzed by inductively coupled plasma mass spectroscopy (ICP-MS; X Series, Thermo Scientific, USA) and flame atomic absorption spectrometry (AAS; Analytik Jena ContrAA 700 HR-CS-AAS), and contained 1034 µg/L of total chromium, 3 µg/L of total copper and 721 µg/L of total arsenic. Two other solutions of CrO_3 and As_2O_5 (analytical reagent grade; Merck, Darmstadt, Germany) were also prepared containing respectively 1034 µg/L of chromium and 721 µg/L of arsenic, with the final pH adjusted to 7.0 (Matos et al., 2009a).

2.2 Animal treatment

Two months old ICR-CD1 male mice (26-40 g) were purchased from Harlan Interfauna Iberica S. A. (Barcelone, Spain). Before experimental use, animals housed in stainless steel cages were allowed to acclimatize for one week under controlled conditions (temperature 22 ± 2 ºC, relative humidity 40-60%, light-dark cycle 12h). Food and water were supplied ad libitum.

Mice were separated until sacrifice into 16 different groups (each with five animals), corresponding to different treatments and times of exposure. Control animals were subcutaneously administrated with the vehicle (0.3 mL of 0.9% NaCl). Exposed groups received a single subcutaneous injection (0.3 mL) of,

respectively As_2O_5 (As 7.2 mg/kg body weight), CrO_3 (Cr 10.2 mg/kg body weight) or CCA (As 7.2 mg/kg body weight and Cr 10.2 mg/kg body weight). This dose was established based on preliminary assays of Matos et al. (2009 a, b) and on LD50 values for Cr and As. Two-hundred-fold dilution of the commercial Type C CCA solution was the lowest concentration that promoted nephrotoxicity with 100% of mice survival. After periods of 14 h, 24 h, 48 and 96 h, animals were sacrificed, and kidneys were removed and fixed in neutral 10% buffered formalin. Samples were then dehydrated and embedded in paraffin wax according to Oliveira et al., (2006b).

Animal trials were conducted in accordance with the guidelines of European Directive for ethics in animal experimentation (2010/63/EU).

2.3 Flow cytometric analyses

Samples were treated as described by Oliveira et al., (2006b) for flow cytometry analyses. Briefly, five block sections of 40 µm thickness of each animal were used per treatment. Sections were initially dewaxed in xylol and rehydrated, then digested for 1h with pepsin pH 1.5 at 37 ºC, and washed in PBS (Oliveira et al., 2006b). Samples were then centrifuged (500 x g) for 10 min and the supernatant was removed. The pellet was washed twice with 1 ml PBS buffer. After filtration (50 µm mesh), 50 µL of RNase (Sigma, St. Louis, MO, USA) was added to 500 µL of sample and the mixture was incubated for 10 min at 37ºC. Finally, 50 µg mL-1 of propidium iodide (PI) was added and the samples were incubated at 4ºC, for 10 min.

The relative properties of the light scattering, SS (side scatter) and FS (forward scatter), and the relative fluorescence intensity of the PI-stained nuclei were measured with a Coulter EPICS XL flow cytometer (Coulter Electronics, Hialeah, Florida, USA). The instrument was equipped with an air-cooled argon-ion laser tuned at 15 mW and operating at 488 nm. The integral fluorescence together with the height and width of the fluorescence pulse from nuclei was collected through a 645 dichroic long-pass filter and a 620 band-pass filter and converted on 1024 ADC channels.

Prior to the analysis, the instrument was checked for linearity with fluorescent check beads (Coulter Electronics, Hialeah, FL) and the amplification was adjusted so that the peak corresponding to the diploid peak was positioned at channel 200. The results were expressed as percentage of nuclei of peak variation, of G0/G1, S and G2/M phase and of HPCV. Sample analysis was done in duplicate.

2.4 Statistical analyses

Statistical analyses were performed using a one-way (ANOVA) (SigmaStat for Windows Version 3.1, SPSS Inc., USA) to compare the different groups. A multiple comparison Tukey test was applied to assay the differences between control and metal treated groups. Correlations between the results obtained with the different treatments were performed by Pearson´s correlation test. In all cases, the level of statistical significance was set at $p \leq 0.05$.

3. Results

To determine the effect of CCA, chromium trioxide and arsenic pentoxide on the cycle of kidney cells was analyzed by the percentage of nuclei in each phase of the cell cycle (G0/G1, S and G2/M). The typical diploid profile of control cells is displayed in Figure 1 while Figures 2 to 4 further illustrate the results for all treatments and periods tested. While CrO_3 had no significant influence on cell cycle dynamics, during the 96 h of exposure (Fig 2a-d), As_2O_5 showed an increase in the percentage of cells in S phase and a decrease in the percentage of cells in G2/M phase after 24h (Figure 3B). After 48h of exposure to As_2O_5, the percentage of cells in G2/M tended to increase (Figure 3C), and after 96h there was a significant increase in the percentage of cells in G0/G1 phase.

Regarding CCA, despite a sudden decrease of cells in S-phase, it was evident that, for longer periods, the cells were delayed in the S (and G2/M) phase, most at expenses of cells in G0/G1 phase. Data presented in Figures 2A and 3A show some similar profiles of response to CCA and As_2O_5, namely regarding

the initial decrease of cells at S phase, followed by an increase of cells in this stage. However, for the G0/G1 phase, the As and CCA effects differed substantially, with only As leading to a blockage of cells at this stage.

Table 1 shows the HPCV values of G0/G1 peaks for As_2O_5, CrO_3 and CCA observed during 14, 24, 48 and 96h.

Table 1. G0/G1 half peak coefficient of variation (HPCV) values observed for control, CCA, CrO_3 and As_2O_5 treated animals.

	Control	**CCA**	**CrO_3**	**As_2O_5**
14h	4.7 ± 1.1	3.9 ± 0.3	4.3 ± 0.8	4.5 ± 1.2
24h	7.8 ± 7.1	4.5 ± 0.4	4.9 ± 0.7	5.1 ± 1.1
48h	6.1 ± 2.7	4.9 ± 1.0	4.4 ± 1.2	3.9 ± 0.5
96h	3.6 ± 0.6	4.1 ± 1.2	4.5 ± 0.5*	4.8 ± 0.7

* means statistically differences ($p<0.05$)

These results showed that treatment with CrO_3 for 96 h induced an increase in the HPCV of cells in G0/G1 phase ($p<0.05$).

4. Discussion

The spread of environmental and occupational pollutants, such as arsenic and chromium contained in wood products treated with CCA, emphasizes the concern of their impact on public and animal health (Barraj et al., 2009, Gress et al., 2014, 2016, Tang et al., 2015). This concern is increased due to the expected increase of CCA-treated wood waste over the next four decades, with demonstrated rise of CCA metals to values above legal limits at EU and USEPA ecological soil screening levels (USEPA, 2003, 2005). In addition, air emissions of combustion of CCA-treated wood in open fires, simulating waste wood

domestic burning, have been reported to exhibit the more toxic trivalent form of As in respirable particle matter fractions (Wasson et al., 2005).

Cr and As may have genotoxic effects, both leading to DNA mutation and/or degradation. Moreover, the effects of Cr and As on cell proliferation have been reported in small intestinal epithelia of mice (Megyesi et al., 1995) and in human keratinocytes (Tse et al., 2009). However, subsequent to induced damage, quiescent cells often can proceed throughout the cell cycle (Matos et al., 2010), a major cause of abnormal cell subpopulations and, eventually, a major source of tumorigenesis. In the kidney, subsequent to acute tubular necrosis, these quiescent cells consist essentially in epithelial cells of the renal tubules (proximal and distal tubules) (Megyesi et al., 1995). In previous experiments, animals treated with CCA and CrO_3 showed remarkable damage in renal tubules (e.g. hyaline and granular casts), demonstrating the high nephrotoxicity of CCA and CrO_3 compounds after 96 h (Matos et al., 2009a). Our previous studies also quantified the levels of arsenic, chromium and copper levels in kidneys of mice exposed to As_2O_5, CrO_3 and CCA (Matos et al., 2009a, Matos et al., 2009b; Matos et al., 2010). For example, in mice exposed to CCA, the levels of Cr observed in the kidney ranged from 424 µg/g at 14 h to 173 µg/g at 96 h. The levels of As were between 32.6 µg/g and 6.54 µg/g for the same periods (Matos et al., 2009a, Matos et al., 2009b, Matos et al., 2010). The levels of Cu were constant (approximately 16 µg/g in all exposure times) (Matos et al., 2010).

A protocol previously developed to release nuclei from paraffin embedded testicles was used (Oliveira et al., 2006b). Embedded material is not widely used in FCM as fresh or fixed material. The protocol showed high reproducibility for renal analysis and showed histograms of FCM with small coefficients of variation and narrow peaks, supporting that this preserved material is suitable for FCM analyses (Oliveira et al., 2006b). In the current work, CrO_3 treated samples showed increases in HPCV of the G0/G1 peak, a parameter correlated with clastogenicity (Rayburn and Wetzel 2002), after 96 h. The different nuclei arresting observed for distinct phases (G0/G1, S and G2/M) of cell cycle promoted particularly by As_2O_5 and CCA treatments, suggests a cytostatic profile gradient of CCA > As > Cr, with CCA and As leading to an accumulation

of S-cells phases, but only As leading to a blockage in G0/G1 phase. Our data support that As and Cr interfere differently with the cell cycle checkpoints, with As leading mainly to a delay in S phase, which may be then aggravated by the combination with Cr in the CCA. Similarly, Chen and Shin (2002) have disclosed that metals and metalloids might affect the expression and function of regulatory proteins at important cell cycle checkpoints. A trend for nuclei arresting at G1 phase, for 14, 24 and 96 h, in the groups of animals treated with As_2O_5 was observed in the present work. This result is in accordance with a previous study in which 10 mM $NaAsO_2$ induced high toxicity to normal and transformed fibroblasts, with G0/G1 phase being the most affected (Waalkes et al., 2000). Waalkes and collaborators (2000) suggested that toxicity of arsenic was due to suppressor tumoral gene (p53), considered a key regulator of cell cycle. P53 is relevant for the modulation of another important protein, p21, a key regulator for the nuclei transition from G0/G1 to S phase (Megyesi et al., 2002). On the other hand, Chen and Shin (2002) observed that trivalent arsenic was a phosphatase inhibitor. Phosphatase is required for the progression of nuclei from the G0/G1 phase to S and G2 phases. Therefore, their inhibition could affect the transition by arresting the nuclei at G0/G1 phase. On the other hand, for animals exposed to As_2O_5 during 24h, a delay in S phase was detected which induced a decrease in the percentage of cells in G2. Tse and co-workers (2008) also found an arrest at G1 in HaCat cells exposed to arsenic pentoxide. As reported above, exposure to CCA increased (48 and 96h) a S phase delay. For these same periods, previous studies by our group (Matos et al., 2009a) demonstrated the presence of acute tubular necrosis in animals exposed to CCA. Megyesi and co-workers (2002) proposed that after damage, the need for repair and/or regeneration of renal cells was based on the cell cycle, and their inhibition allows them to repair cell injuries prior to all replication. As shown for animals exposed to CrO_3, As and CCA, the apparent tendency for a cell cycle arrest at G2 phase at 48 h (although not statistically significant, $p>0.05$) returned to the control values at 96 h. A transient delay in G2 phase was also observed by McCabe and co-workers (2000) in leukemia cells exposed to arsenite. These authors found that while G1 and G2 phases appear to be delayed by As, cells in G2 phase seem to be more sensitive to arsenite.

In conclusion, we report here that CCA and its individual components, chromium and arsenic have different effects on mouse kidney cell cycle dynamics. CCA and As compounds exhibited clastogenic effects, particularly in the S phase. The kidney, being a detoxifying organ, is a main target for CCA and its individual components. Although there are already some technologies to identify and remove CCA wood from wood recycling streams (Robey et al., 2018; Jones et al., 2019; Kim et al., 2019), and considering the demonstrated toxicity of CCA and its constituents in renal cell genotoxicity, the authors emphasize the need for continuous and effective management of these hazardous products to protect public health.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships
that could have appeared to influence the work reported in this paper.
☐The authors declare the following financial interests/personal relationships which may be considered
as potential competing interests:

5. Conflict of interest

The authors declare that there is no conflict of interest.

6. Acknowledgements

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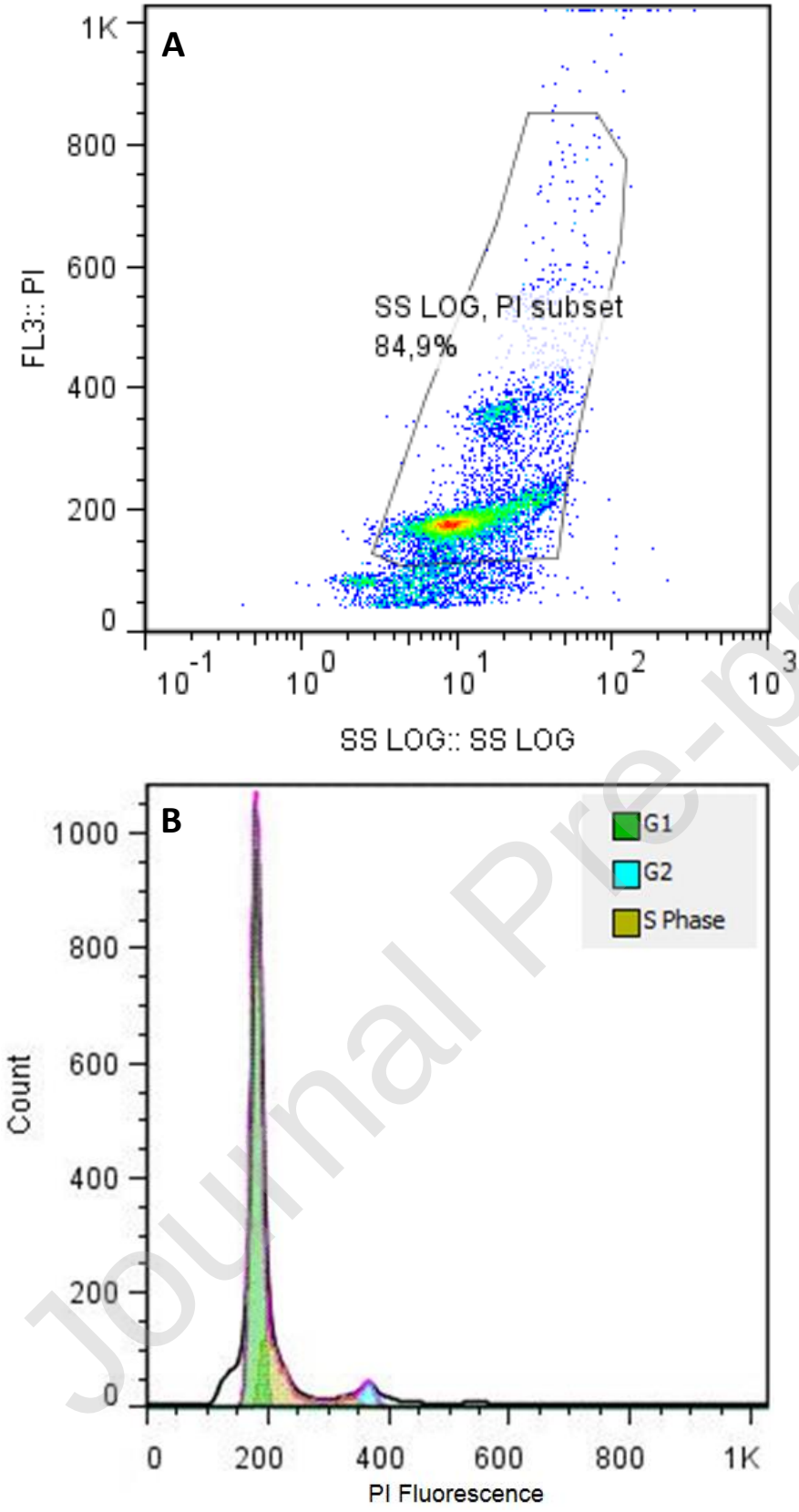

Figure 1 Typical DNA frequency histogram of control deparaffinized mice renal cells (see text for details). (A) Cytogram of relative fluorescence light intensity from PI versus side angle light scatter (SS). Outside the close polygonal line are artifacts (i.e., 15.1 % of debris, doublets and

aggregates) electronically excluded form analysis. (B) Cell cycle histogram representing the linear fluorescence of the DNA stained with PI, showing calculated G_0/G_1, S and G_2/M phases.

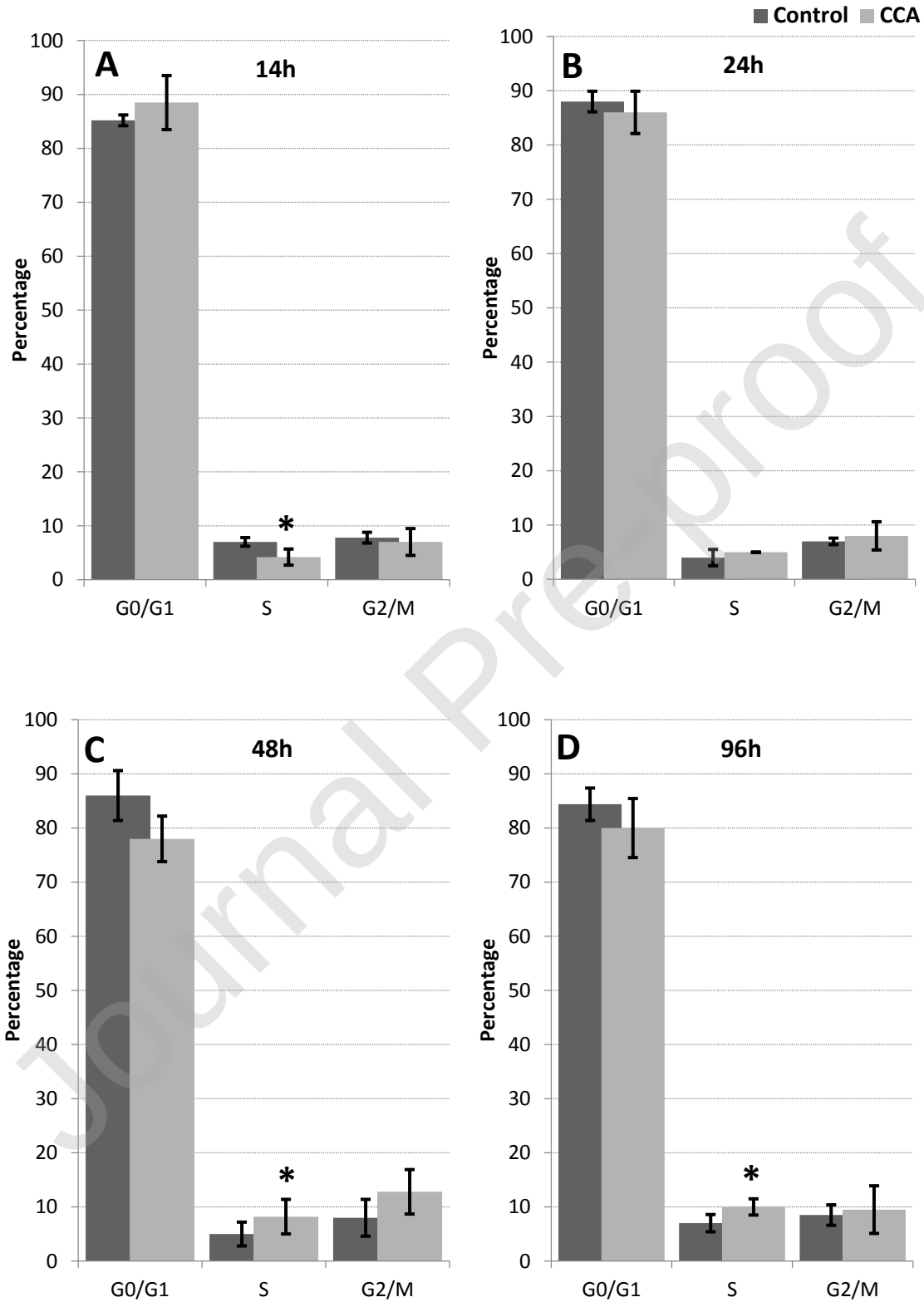

Figure 2 - Cell cycle analysis for control and CCA treated animals for the periods of (A) 14h (B) 24h (C) 48h and (D) 96h. * means statistically differences (p<0.05)

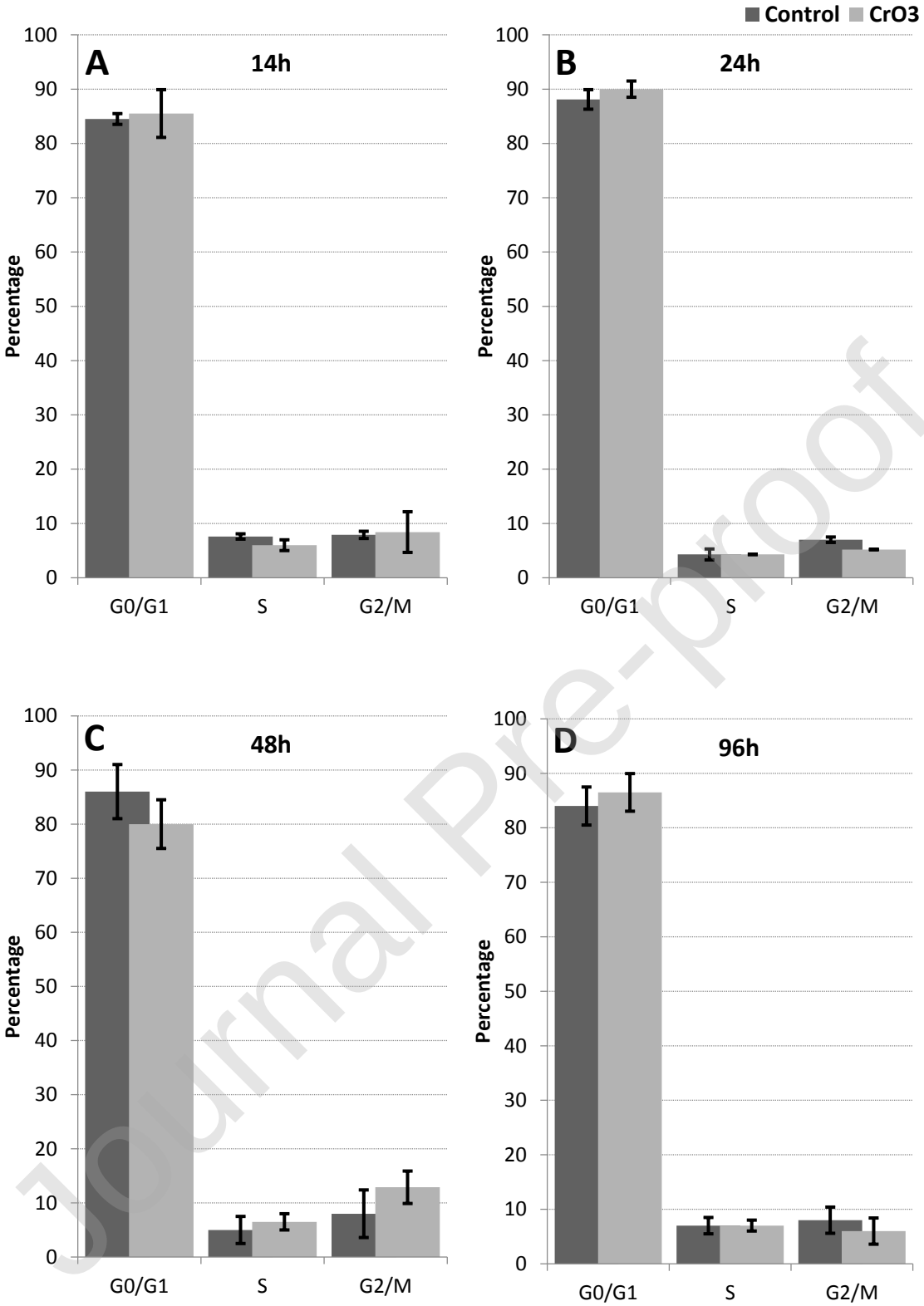

Figure 3 Cell cycle analysis for control and CrO_3 treated animals for the periods of (A) 14h (B) 24h (C) 48h and (D) 96h.

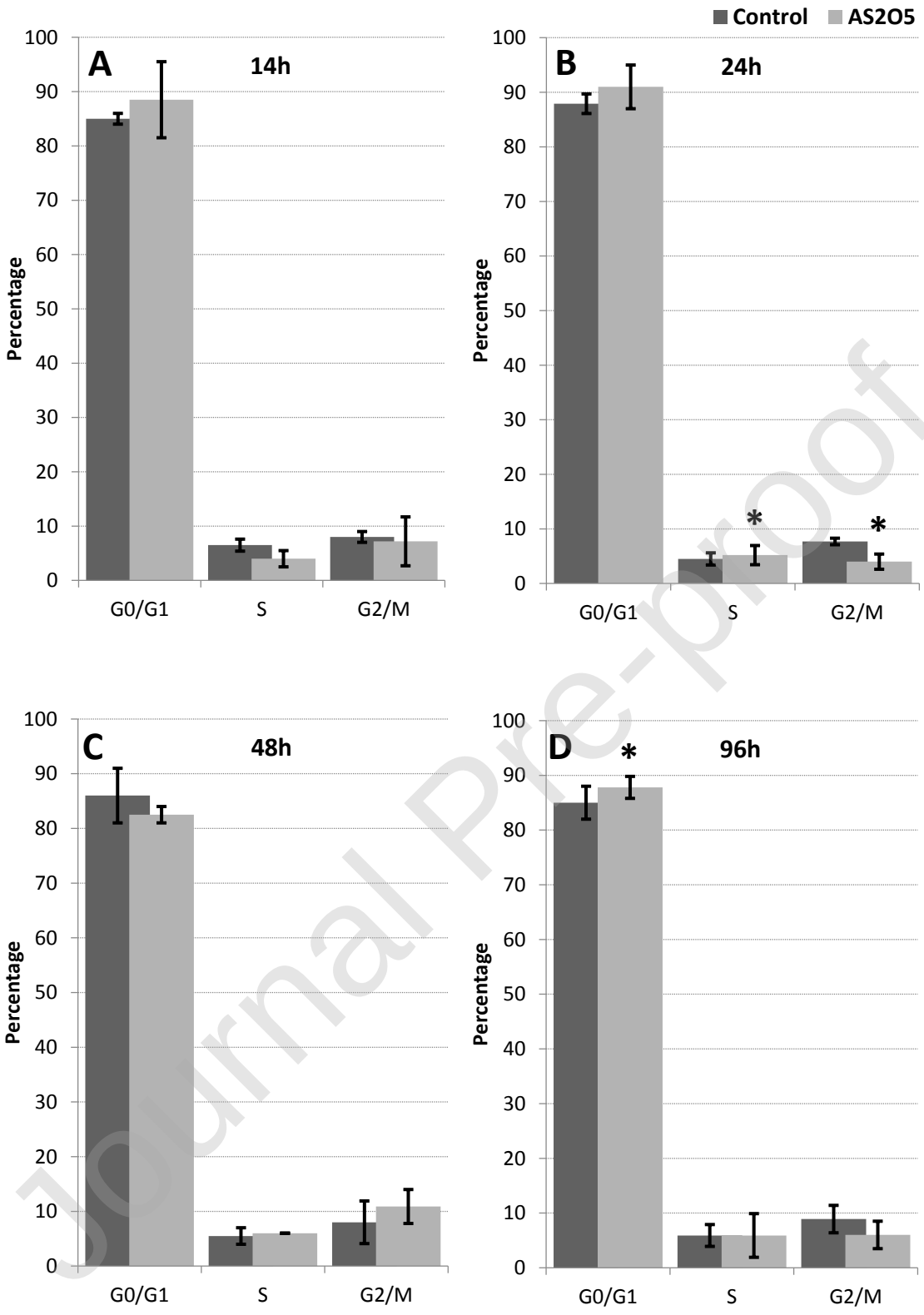

Figure 4 Cell cycle analysis for control and As_2O_5 treated animals for the periods of (A) 14h (B) 24h (C) 48h and (D) 96h. * means statistically differences (p<0.05).