SILVIA TAMIE MATSUMOTO

Estudos sobre a influência de efluentes potencialmente genotóxicos, derivados de curtume, na contaminação de recursos hídricos da região de Franca, SP

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Programa de Pós-Graduação em Genética

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Tese apresentada para obtenção
Do Título de Doutor em Genética.

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Aos meus pais (*in memorian)*,
Fernanda, Haruka, Tico e Yuta.

"Deus nos fez perfeitos e não escolhe os capacitados, capacita os escolhidos. Fazer ou não fazer algo só depende de nossa vontade e perseverança."

(Albert Einstein)

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RESUMO

Os agentes mutagênicos são substâncias que induzem alterações na molécula de DNA, que podem ou não serem corrigidas pelo próprio mecanismo de reparo das células. Alterações não reparadas, ou reparadas erroneamente, originam mutações de ponto e/ou cromossômicas. Os metais pesados são potencialmente mutagênicos. Nos últimos anos, tem aumentado muito a emissão de metais pesados, causando impacto severo nos ecossistemas e um comprometimento grave à saúde humana. Os cromos trivalente (III) e hexavalente (VI) diferem entre si principalmente pelas diferentes habilidades que possuem quanto ao modo de transporte através de membranas biológicas. O Cromo VI apresenta uma estrutura geométrica tetraédrica, que é menor e, portanto, mais permeável às membranas biológicas do que o cromo III, o qual apresenta uma estrutura geométrica octaédrica. Foi investigado, por meio de ensaios biológicos com diversos organimos teste, a possível presença de agentes contaminantes, com potencial citotóxico, genotóxico e clastogênico, que são despejados no Rio Sapucaizinho, Município de Patrocínio Paulista/SP e Córrego dos Bagres, Município de Franca/SP, onde se encontra várias indústrias de curtimento do couro (curtume). Foram realizados teste de indução de aberrações cromossômicas em *Allium cepa*, de micronúcleos em *Oreochromis niloticus,* e ensaio do cometa em *Oreochromis nioticus* e em células de ovário de

Hamster (CHO-K1). As análises revelaram que os organismos teste submetidos aos tratamentos com águas contaminadas com cromo apresentaram uma maior indução de genotoxicidade em suas células. Foi observada uma maior indução de alterações nas amostras de água derivadas de coletas em local de despejo de efluente de curtume, seguida pelas águas do local imediatamente abaixo ao despejo. Pelas análises químicas realizadas, podemos observar que esses locais são os que apresentam uma maior contaminação por cromo, de onde se pode concluir que esse metal seja o principal responsável pelas alterações celulares encontradas. Os efeitos citotóxicos e genotóxicos do cromo, observados em organismos teste, são detectáveis para concentrações de 0.01 mg/L de cromo, concentração essa muito inferior à permitida pela Legislação do Estado de São Paulo, que é de 0.05mg/L.

Palavras-chave: *Allium cepa*; CHO-K1; Ensaio do cometa; Genotoxicidade.

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ABSTRACT

Mutagenic agents are substances that induce changes in the DNA molecule that can be or cannot be corrected by repair mechanism of the cells themselves. Unrepaired or erroneously repaired changes give origin to point and/or chromosomal mutations. Heavy metals are potencially mutagenics. During the last years the emission of metals has increased, causing a severe impact to the ecosystems and seriously compromising human health. Trivalent chromium (III) and hexavalent chromium (VI) differ from one another in their ability to cross biological membranes. Cr VI has a tethraedrical geometric structure, that is smaller and therefore, is more permeable to the biological membranes than chromium III, that has an octahedral geometric structure. It was investigated, through biological assays with different test organisms, the possible presence of contaminant agents with citotoxic, genotoxic and clastogenic potentials that are poured in the Sapucaizinho River, Municipality of Patrocínio Paulista/SP and in the "Córrego dos Bagres"stream, Municipality of Franca/SP, where there are intense industrial activity related to leather processing (tanning). It was realized the induction of chromosome aberrations test in *Allium cepa*, the micronucleus test in *Oreochromis niloticus* and comet assay in *Oreochromis niloticus* and in Hamster's ovary cells (CHO-K1). The analysis revealed that the test organisms that were submitted to the treatment with chromium contaminated

cm 1 2 3 4 5 6 7 8 unesp 12 13 14 15 16 17 18

water presented a higher induction of genotoxicity in their cells. A higher induction of alterations was observed in the water samples derived from collections in the tannery effluent discharge site, followed by the water of the site immediately downstream the discharge. By the chemical analysis made, we could observe that these sites are the ones that present a biggest chromium contaminations, where it could be concluded that this metal is the principal responsable of the cellular alterations found. The chromium citotoxic and genotoxic effects, observed in the test organisms, are detectable for the 0.01mg/L chromium concentration, which is much lower than the concentration permitted by the São Paulo State legislation, that is 0.05mg/L.

Key words: *Allium cepa*; CHO-K1; Comet assay; Genotoxicity

1. - INTRODUÇÃO

1.1 – Mutagênese ambiental

A genética ecotoxicológica investiga os mecanismos de ação de agentes toxicantes que são capazes de induzir interações altamente específicas com os ácidos nucléicos, podendo resultar em danos genéticos, como mutação de ponto, erros durante o mecanismo de replicação do DNA, irregularidade mitótica, dentre outros.

Os agentes mutagênicos são substâncias que induzem alterações na molécula de DNA, que podem ser corrigidas pelo próprio mecanismo de reparo das células. Alterações não reparadas, ou reparadas erroneamente, originam mutações de ponto e/ou cromossômicas (CONNOR e FERGUNSON, 1993). As alterações cromossômicas, tais como quebras e "gaps", são normalmente visualizadas em cromossomos metafásicos. Na anáfase, os tipos aberrantes encontrados são geralmente quebras, perdas, pontes cromossômicas e multipolaridades. As quebras podem ser simples ou aos pares, e as pontes podem ser simples ou duplas (NATARAJAN e OBE, 1982; PAVLICA et al., 2000; MATSUMOTO et al., 2003).

A maioria das substâncias químicas com potencial genotóxico é capaz de alterar o material genético em todas as fases do ciclo celular, porém, para se fixarem como alterações cromossômicas, necessitam de mecanismo de replicação do DNA, acontecido logo após a ocorrência dessas alterações (OBE et al. 1982). Muitos agentes mutagênicos induzem efeitos sobre

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núcleos eucromáticos, que podem ser detectados citologicamente pela inibição e interrupção do progresso da divisão celular, aberrações cromossômicas numéricas e estruturais, trocas de cromátides irmãs e outros.

Pesquisas que investigam a ação de agentes mutagênicos ambientais vêm tendo grande importância, pois sabe-se que, embora ocorram mutações espontâneas, a maior parte delas é induzida por agentes físicos, químicos ou biológicos, aos quais o homem pode estar exposto.

Muitas técnicas estão sendo desenvolvidas para avaliar o potencial mutagênico de agentes poluidores, tais como: análise de aberrações cromossômicas; análise de DNA *fingerprinting*; análise da freqüência de quebras na molécula de DNA; ensaio do cometa; quantificação dos adutos de DNA e teste de micronúcleo (McCARTHY et al., 1989; DEPLEDGE, 1996; MATSUMOTO et al., no prelo).

Os testes citogenéticos permitem a compreensão dos efeitos de algumas substâncias sobre os organismos teste e mostram ser eficientes quando utilizados no biomonitoramento da extensão da poluição (MORAES, 2000).

Existem muitas categorias de classificação das fontes de poluição ou das atividades humanas que alteram a qualidade da água. Dentre elas, temos as atividades industriais, a produção de energia, o transporte, a mineração, a contaminação de aqüíferos por atividade agrícola e/ou a atividade humana (FALKENMARK e ALLARD, 1991). Já as substâncias

poluidoras são classificadas em diferentes categorias como: ácidos e agentes acidificantes (SO_2, NO_n); nutrientes (NO_3^-, PO_4^{-3}); compostos orgânicos degradáveis; metais e metalóides; outros (FALKNMARK e ALLARD, 1991).

Bioensaio com *Salmonella* e/ou o teste de Ames são freqüentemente utilizados para avaliação de genotoxicidade. Desde o advento destes testes, muitas outras análises têm sido utilizadas para avaliar a genotoxicidade, sendo o DNA o alvo de estudo (WHITE et al., 1996), que segundo Houk (1992) pode acontecer de modos diferentes, configurando três grandes classes de danos: **mutagênico**: referente à mutação no gene (ou de ponto); **clastogênico**: referente à alteração na estrutura cromossômica; e **aneuploidia**: referente à perda ou ganho de cromossomos inteiros.

O teste de micronúcleo tem sido um dos testes mais promissores para a avaliação de genotoxicidade, pela sua eficiência, simplicidade e rapidez, sendo utilizado pela primeira vez por Evans et al. (1959 *apud* FERRARI, 1991), como um indicador de dano citogenético. Os autores utilizaram a freqüência de micronúcleo para avaliar o dano induzido em raiz de cebola por nêutrons e raios-gama. A ocorrência de micronúcleo tem sido utilizada como indicador de efeitos clastogênicos (quebras no DNA) causados por agentes químicos e físicos (WALKER et al., 1996).

O micronúcleo apresenta estrutura similar ao núcleo principal, tendo também o seu DNA envolto por membrana nuclear. É encontrado no

citoplasma e não apresenta nenhuma relação com o núcleo principal (WALKER et al., 1996). Sua origem se dá durante a divisão celular, decorrente de atraso cromossômico na anáfase, ou por fragmentos cromossômicos. Os micronúcleos podem surgir espontaneamente ou em resposta à exposição de agentes genotóxicos sobre os cromossomos (FRIEAUFF et al., 1998). Como a formação do micronúcleo se dá durante os processos de divisão celular, esses micronúcleos só serão realmente visualizados em células que tenham completado a primeira mitose, após ter sofrido uma exposição ao agente genotóxico, sem que, no entanto, tenham iniciado sua segunda divisão (FALCK et al., 1997).

O uso de bioensaios é eficiente para a identificação de toxicidade aguda. Desta forma, a toxicidade aguda apresenta, segundo White et al. (1996), o primeiro nível de impacto no ecossistema aquático. Os autores expõem que, atualmente, muitas descargas industriais contêm muitas substâncias que, mesmo não tendo efeito agudo, são capazes de reduzir, em longo prazo, a sobrevida de um organismo. A redução de sobrevida se deve aos danos causados no genoma das células somáticas e/ou germinativas. Esses danos genéticos têm sido relacionados às desordens genéticas e ao câncer.

Testes biológicos para toxicidade e genotoxicidade são, segundo Moraes (2000), indispensáveis para a avaliação de reações de organismos vivos à poluição ambiental complexa e para uma indicação dos efeitos

sinergísticos potenciais de vários poluentes, enquanto análises físico-químicas identificam a presença e as respectivas concentrações de diferentes poluentes.

Dentre os vegetais superiores, *Allium cepa* tem sido indicado como um eficiente organismo-teste de citotoxicidade e genotoxicidade (FISKEJO, 1985), devido às características que possui na sua cinética de proliferação, pelo crescimento rápido de suas raízes, pelo grande número de células em divisão, pela sua alta tolerância a diferentes condições de cultivo, pela sua disponibilidade durante o ano todo, pelo seu fácil manuseio, e por possuir cromossomos em número reduzido (2n=16) e de grande tamanho (QUINZANI-JORDÃO, 1987).

O uso de *Allium cepa* como material teste foi originalmente introduzido por Levan (1949), e a partir daí tem sido utilizado para avaliar e classificar a toxicidade de químicos presentes no meio ambiente (FISKEJO, 1985). Essa espécie tem sido utilizada como material vegetal teste indicador de mutagênese por diversos autores como Khors et al. (1997), Rank e Nielsen (1997), Smaka-Kincl et al. (1997) e Kovalchuck et al. (1998). Testes com *Allium cepa* são adequados por oferecer parâmetros macroscópicos como turgescência, mudança de cor, formato, textura, espessura, comprimento da raiz e parâmetros microscópicos como anáfases prematuras, aderências cromossômicas, pontes e fragmentação cromossômica, C-mitoses e

micronúcleos, que são indicadores de eventuais mutações no conteúdo genético celular (QUINZANI-JORDÃO, 1987).

Várias técnicas, na tentativa de avaliar os danos na molécula de DNA, têm sido desenvolvidas nos estudos dos efeitos mutagênicos das substâncias tóxicas. Assim, dependendo da mutação a ser investigada, e do tipo do material a ser avaliado, é realizada a escolha do organismo teste mais adequado ao estudo. A utilização de células de mamíferos, como organismo teste, tem sido aceitável na tentativa de relacionar os resultados da ação dos agentes contaminantes nessas células, com os possíveis efeitos, desses agentes, no homem (RABELO-GAY et al., 1991).

Culturas de células de mamíferos são utilizadas, com sucesso, nos estudos de avaliação da mutagenicidade dos agentes tóxicos. Esses testes apresentam vantagens pela facilidade na padronização das condições do ensaio, possibilidade de realizar tratamentos das células em várias fases do ciclo celular, e pela organização do material genético ser igual às células *in vivo* (RABELO-GAY et al., 1991).

Dentre os mamíferos, células de CHO-K1 (ovário de hamster chinês) são indicadas para a análise da indução de danos no DNA. A vantagem da utilização dessas células é por apresentarem um número reduzido de cromossomos (2n=20), facilidade de crescimento em cultura, e por apresentarem ciclo celular relativamente curto (10 a 14 horas). Porém, apresentam algumas desvantagens na avaliação de aberrações

cromossômicas, pois podem formar populações celulares não-sincrônicas, com considerável heterogeneidade no ciclo celular e também por serem células transformadas (PRESTON et al., 1987).

O ensaio do cometa tem sido proposto como uma ferramenta no monitoramento da genotoxicidade de águas, que utilizam peixes ou culturas de células de CHO-K1, para detecção de danos no DNA induzidos por mutágenos diretos e pró-mutágenos dissolvidos na água (OLIVE et al., 1992; ANDERSON e PLEWA, 1998; MATSUMOTO et al., 2003).

O ensaio do cometa consiste em uma metodologia, que investiga quebras de fita de DNA ou sítios álcali-lábeis, e pode servir como bioindicador de genotoxicidade em vários organismos. Esses biomarcadores são importantes para a avaliação da potencialidade do carcinógeno, do efeito sobre o sistema reprodutivo dos organismos e outros efeitos adversos da poluição. É um método sensível e rápido para mensurar o dano e o reparo do DNA em células individuais, podendo ser esse dano evidenciado por migração em eletroforese (OLIVE et al., 1992; KLAUDE et al., 1996; MITCHELMORE e CHIPMAN, 1998; LEE e STEINERT, 2003).

Para a realização do ensaio do cometa, diferentes tipos de organismos, de tecidos e de células podem ser expostos a agentes contaminantes, *in vivo* ou *in vitro* para que se possa avaliar os danos na molécula de DNA provocados pelos agentes (RAJAGURU et al., 2002;

GONTIJO et al., 2003; LEE e STEINERT, 2003; OHE et al., 2003; MATSUMOTO et al., no prelo).

1.2 - Genotoxicidade dos metais pesados

Todos os organismos vivos estão em interação com o meio ambiente. Assim, o seu genoma fica exposto às interferências que esse meio sofre. A interação entre o meio e o organismo resulta em modificações que, quando positivas, refletem na adaptação do organismo quanto à melhor exploração desse meio, o que decorre também na própria modificação do ambiente pelo organismo (MINISSI e LOMBI, 1997; PASCALICCHIO, 2002).

Nos últimos anos, a emissão de metais pesados no ambiente tem aumentado, causando impacto severo nos ecossistemas e comprometimento grave à saúde humana (SNOW, 1992; WÜRGLER e KRAMERS, 1992; MAJER et al., 2002). As principais atividades responsáveis pela contaminação por metais pesados em solos e águas são por atividades industriais, agrícolas e urbanas, decorrendo em sérios problemas ambientais (BRIAT e LEBRUN, 1999). Segundo Pascalicchio (2002), além das atividades industriais, emissões naturais também podem estar relacionadas com a contaminação ambiental por metais pesados.

Os metais pesados são potencialmente mutagênicos e estão seriamente envolvidos com a poluição ambiental. Alguns metais pesados induzem, comprovadamente, a formação de tumores em organismos

experimentais e em seres humanos eventualmente expostos (SUNDERMAN, 1984; FRIBERG et al., 1985).

Os metais Se, Cd, Cr e Ni são encontrados normalmente na alimentação, em quantidades baixas, mas quando em excesso, são considerados carcinogênicos e/ou mutagênicos (MINISSI e LOMBI, 1997).

Os derivados de metais e seus metabólitos induzem, tanto *in vivo* como *in vitro*, alterações nas estruturas dos cromossomos, promovendo mutações de pontos em procariotos e eucariotos (PASCALICCHIO, 2002).

Os organismos estão comumente expostos a agentes oxidantes. Esses agentes oxidantes, quando em excesso, podem causar danos às moléculas biológicas, como, por exemplo, nos lipídios, proteínas, carboidratos e DNA (SIES, 1993; HALLIWELL et al., 1995). Os metais pesados podem estar relacionados com a produção destes agentes oxidantes (STOHS e BAGCHI, 1995).

Compostos de metais pesados têm se mostrado carcinogênicos, podendo afetar o desenvolvimento e o crescimento de alguns animais (DE et al., 1990; SUNDERMAN et al., 1995). A biotransformação de metais pode ser explicada pela interferência da função normal dos metais, com a capacidade de interação com sítios específicos de elementos essenciais, prejudicando o metabolismo basal da célula e, conseqüentemente, o metabolismo do organismo.

Segundo Beyersmann et al (1994), o metal pesado cádmio age no metabolismo celular e sua presença está relacionada com a alteração do balanço entre diferenciação e proliferação celular. O cádmio tem mostrado efeito em vários níveis de transdução celular, como: sinais da membrana plasmática; aumento do cálcio intracelular livre, que interfere no metabolismo da ATPase de vesículas celulares; ativação e translocação de proteína, que pode ativar a promoção tumoral; ativação da expressão oncogênica, pela indução de transcrição de oncogenes.

Durante o metabolismo celular, são produzidas formas reduzidas de oxigênio, que podem ser catalisadas por metais pesados, gerando os radicais livres, que causam alterações nas macromoléculas, o que contribui com o evento de morte celular (BRIAT e LEBRUN, 1999).

Os compostos oxidantes como os peróxidos de hidrogênio (H_2O_2), superóxidos (O_2•-) e os radicais hidroxilas (OH•-), são classificados como espécies reativas de oxigênio (ROS). As ROS, exceto H_2O_2, são consideradas radicais livres, por apresentarem um elétron não pareado em suas estruturas atômicas (HALIWEEL e GUTTERIDGE, 1985). A redução de um elétron, na molécula de oxigênio, para a formação de um radical superóxido (O_2•-), é termodinamicamente desconhecida.

Os metais pesados em transição apresentam, freqüentemente, um elétron não pareado, o que confere a propriedade de serem esses metais bons catalisadores de redução de O_2.

$$METAL^{n} + O_2 \rightarrow METAL^{n+1} + O_2\bullet-$$

^{n} = não pareado

$^{n+1}$ = pareado

Reação de 0_2 com o metal pesado na formação de ROS

Em solução aquosa e pH neutro, o $O_2\bullet-$, gerado segundo a reação acima esquematizada, pode reagir com o H_2O_2 e produzir um radical hidroxila ($OH\bullet-$). Esse radical hidroxila pode danificar moléculas biológicas, levando, em último caso, à morte celular (BRIAT e LEBRUN, 1999). A reação entre $H_2O_2 + O_2$ é chamada de Haber-Weiss, conforme esquema abaixo:

$$O_2\bullet- + H_2O_2 \rightarrow O_2 + OH- + OH\bullet$$

Reação de Haber-Weiss

Quando o ferro é o metal em transição na reação Haber-Weiss, a reação é chamada de Reação de Fenton (HALLIWELL, 1990), conforme descrição abaixo:

$$Fe\ III + O_2\bullet- \rightarrow Fe\ II + O_2$$

$$Fe\ II + H_2O_2 \rightarrow Fe\ III + OH\bullet + OH-$$

Reação de Fenton

Radicais hidroxilas podem ser produzidos próximos à molécula de DNA, podendo adicionar ou remover átomo de H nas bases desse DNA (PRYOR, 1988). Essa reação pode ser responsável por modificações das bases do DNA da célula, da ordem de 10^4 a 10^5 por dia (AMES et al., 1991).

Íons de metais que participam da reação de Haber-Weiss também exercem função importante na modificação oxidativa de aminoácidos livres e proteínas (STADTMAN, 1993).

Os metais pesados podem se ligar às moléculas dispostas no núcleo celular, através das reações químicas que geram as ROS e, conseqüentemente, podem levar a alterações como: modificações nas bases de DNA; ligações entre o DNA e a proteína inter e intramolecular; rearranjos e depurinação (BRIAT e LEBRUN, 1999).

A toxicidade de um dado metal para vários tecidos pode ser explicada pela sua atuação quando em transição. Esses metais atuam como catalisadores na deteriorização oxidativa das macromoléculas biológicas, produzindo ROS, resultando assim na peroxidação lipídica e na alteração do DNA (STOHS e BAGCHI, 1995). O_2 e metal (em transição), em particular o ferro, estão envolvidos na peroxidação dos lipídios da membrana biológica, rica em polissacarídeos e ácidos graxos (SCHOLZ et al., 1990).

A metilação do metal por espécie reativa de oxigênio, nas proximidades do DNA, gera principalmente, aduto promutágeno 8-OXO-G (7,8-hihidro-8-oxoguanina). Esses adutos podem emparelhar-se com a base

A, que, quando na ausência de reparo desse DNA, resultará em mutação do tipo transversão de bases G para T (CUNNINGHAM, 1997) (Figura 1).

Alleman et al. (1985) e Minotti e Aust (1987) relataram a participação do Fe II e Fe III no processo de iniciação da peroxidação lipídica, gerando as ROS, demonstrando ser o Fe, um bom catalisador no processo de formação dessas ROS. O ferro é capaz de catalisar a reação de redução entre oxigênio e macromoléculas biológicas. Quando há ausência de Fe, essa redução não acontece.

O complexo formado por Fe mais Adenosina 5´-difosfato (ADP), histidina, EDTA (Ethlyenedi-aminetetracetic-acid), citrato e outros, facilita a formação de ROS e aumenta a produção de peroxidação lipídica (AUST, 1989; RYAN e AUST, 1992).

As espécies reativas do O_2, tais como H_2O_2 e $O_2\bullet^-$, são geradas, *in vivo*, em todas as células aeróbicas. Em condições fisiológicas, essas espécies reativas não reagem com o DNA, a menos que os íons metálicos estejam presentes para permitir a formação de $OH\bullet$, uma espécie altamente prejudicial ao organismo (BLAKELY et al., 1990).

As reações catalisadas por íons dos metais Fe e Cu parecem ser mais importantes, *in vivo*, para reagir com $O_2\bullet^-$.

Diversos xenobióticos podem ser convertidos em ROS por processos metabólicos intracelulares. Dentre eles, estão os poluentes presentes no ar,

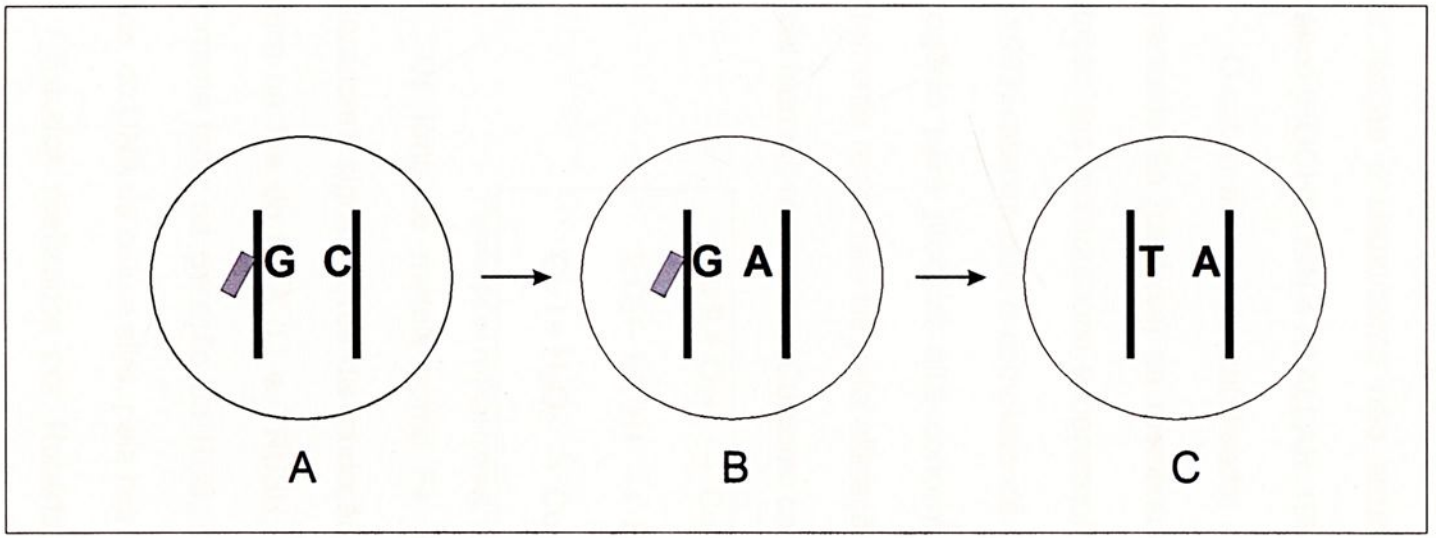

Figura 1 - **A**- aduto ligado a base G; **B**- pareamento da A com G pela modificação da característica da G; **C** - nova síntese de DNA com substituição de base.

pesticidas e certos compostos presentes em alimentos e cigarros (BLOCK, 1992). A auto-oxidação desses xenobióticos forma os peróxidos, sendo que essa reação é usualmente não enzimática, mas dependente de um íon metálico (HOCHSTEIN e ATALLAH, 1988).

O cobre atua como catalisador nos processos de formação da ROS, na peroxidação (catálise) da membrana lipídica (CHAN et al., 1982),e na oxidação das hidroquinona e benzoquinona (LI e TRUSH, 1993). Ozawa et al. (1993) relatam que o complexo de Cu II pode reagir com o peróxido de hidrogênio para produzir alta concentração de radicais hidroxil, que são diretamente responsáveis pela alteração no DNA. A reação de formação do radical hidroxil, utilizando o Cu como catalisador no processo, segue:

$$Cu\ II + O_2\bullet^- \rightarrow Cu\ I + O_2$$

$$2\ O_2\bullet^- + 2\ H^+ \rightarrow H_2O_2 + O_2$$

$$Cu\ I + H_2O_2 \rightarrow Cu\ II + OH\bullet + OH^-$$

Formação do radical hidroxil, utilizando Cu como catalizador

Os íons de metais como Fe III, Mn III, Cd II e Zn II não são aceleradores significativos da oxidação de hidroquinona ou da indução de quebra na fita do DNA (LI e TRUSH, 1993). Entretanto, o Cu exerce um importante fator na geração da ROS, na citotoxicidade, e na promoção de danos do DNA na célula alvo, pela hidroquinona.

Estudos realizados por Robertson (1985) e L'Huillier et al. (1996) mostraram que o acúmulo de níquel nas raízes de milho reduziu a atividade

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mitótica dos meristemas da planta, provavelmente pela perda da integridade do meristema da raiz. Chakravarty e Srivastava (1992), estudando *Helianthus annuus*, descreveram que, dependendo da concentração e do tempo de exposição, o alumínio, o cádmio, o cobre e o níquel promovem um aumento do efeito clastogênico nesta planta.

Muitos organismos testes são utilizados para avaliar os efeitos dos danos no DNA, em estudos de biomonitoramento de solos contaminados por metais pesados. O teste de genotoxicidade convencional, que utiliza bactéria como organismo teste, não tem mostrado ser um bom indicador de danos (ROSSMAN, 1994). Segundo Magos (1991), linhagens de células de mamíferos respondem de forma mais eficiente na avaliação dos efeitos dos danos causados por metais pesados. Estudos mostram que as plantas, em particular a *Tradescantia*, são apropriadas para realizar investigações de genotoxicidade de solos contaminados com metais pesados, por serem altamente sensíveis a esses metais (KNASMÜLLER et al., 1998; STEINKELLNER et al., 1998; MAJER et al., 2002).

1. 3 - Genotoxicidade do cromo (Cr)

O composto de cromo tem efeito tóxico, genotóxico, mutagênico e carcinogênico para o homem e animais (VON BURG e LIU, 1993; STOHS e BAGCHI, 1995; MOUNT e HOCKETT, 2000). Segundo Von Burg e Liu (1993), o cromo promove toxicidade aguda, toxicidade crônica,

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neurotoxicidade, toxicidade reprodutiva, genotoxicidade, carcinogenotoxicidade, além de contaminação ambiental.

Estudos epidemiológicos demonstram que trabalhadores de indústrias de produção do cromato têm uma maior predisposição ao desenvolvimento de câncer de pulmão. No Japão e nos Estados Unidos foi relatado um alto índice de câncer sinonasal em trabalhadores industriais de produtos primários de cromato (ROJAS et al., 1999).

Os cromos trivalente III e hexavalente VI são estado oxidativo ativo em seres vivos. Esses dois estágios de oxidação do cromo diferem, em relação às suas atividades celulares, principalmente devido às diferentes habilidades que possuem quanto à facilidade de transporte através de membranas biológicas (LEONARD e LAUWERYS, 1980; STOHS e BAGCHI, 1995). O Cromo VI apresenta uma estrutura geométrica tetraédrica, que é menor e, portanto, mais permeável às membranas biológicas do que o cromo III, o qual apresenta uma estrutura geométrica octaédrica (Figura 2).

Estudos realizados por Debetto et al. (1982) sugerem que não apenas o Cr III reage diretamente com o DNA, mas que o Cr VI pode ter um mecanismo indireto na produção de genotoxicidade, os quais geram os intermediários de oxigênio reativos. Vários estudos, *in vivo* e *in vitro*, mostram que o Cr VI induz uma variedade de danos à molécula de DNA, incluindo quebras na cadeia principal (Figura 3), e oxidação de bases que geram aberrações cromossômicas formação de micronúcleo (MN), trocas de

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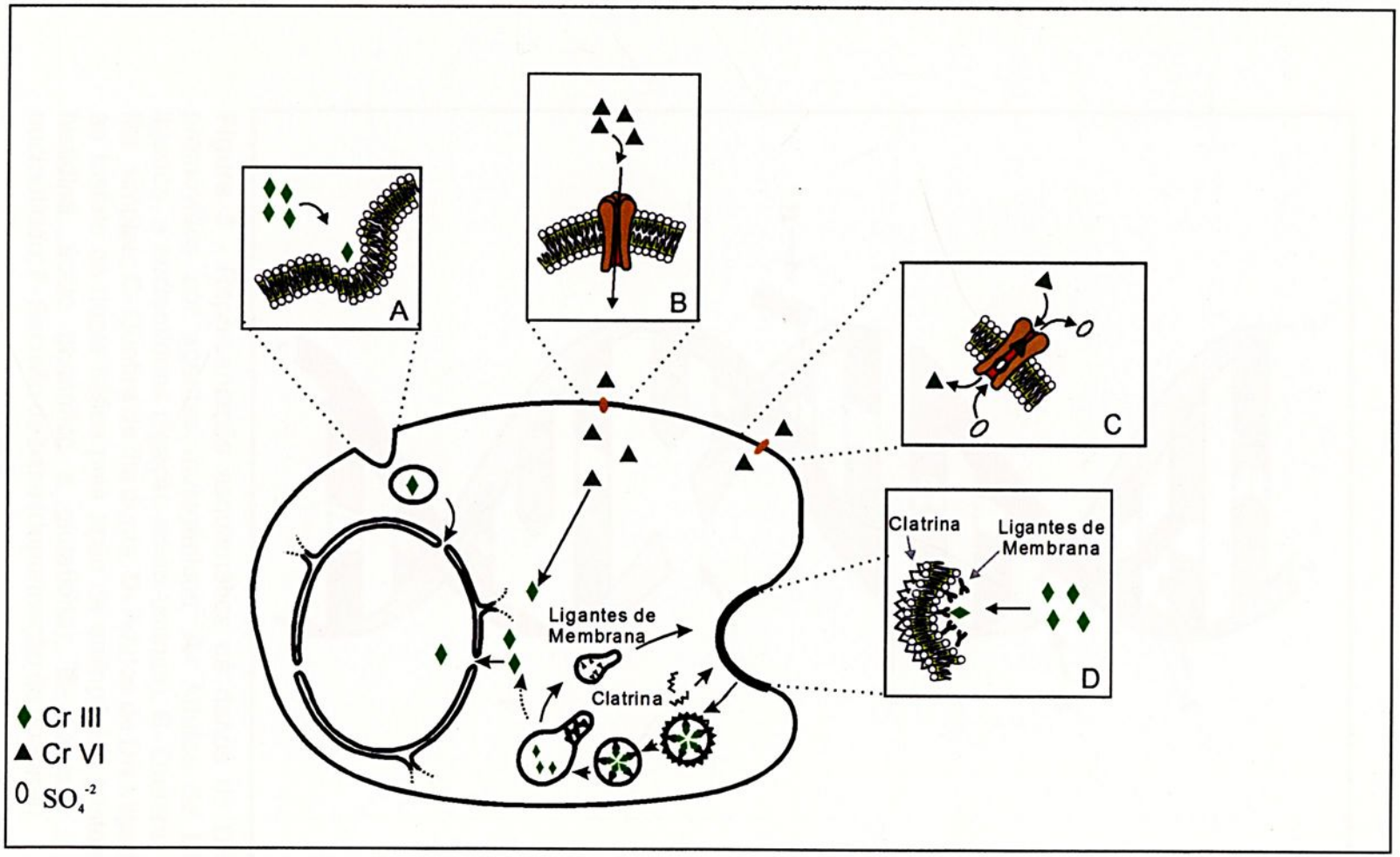

Figura 2 - Vias de transporte transmembrana do cromo III e VI. **A-** Processo de introdução do cromo III na célula (fagocitose); **B-** Transporte passivo do cromo VI, mediado por porteína; **C-** Transporte ativo do cromo VI, mediado por proteína tipo antiporte; **D-** Transporte do cromo III por endocitose.

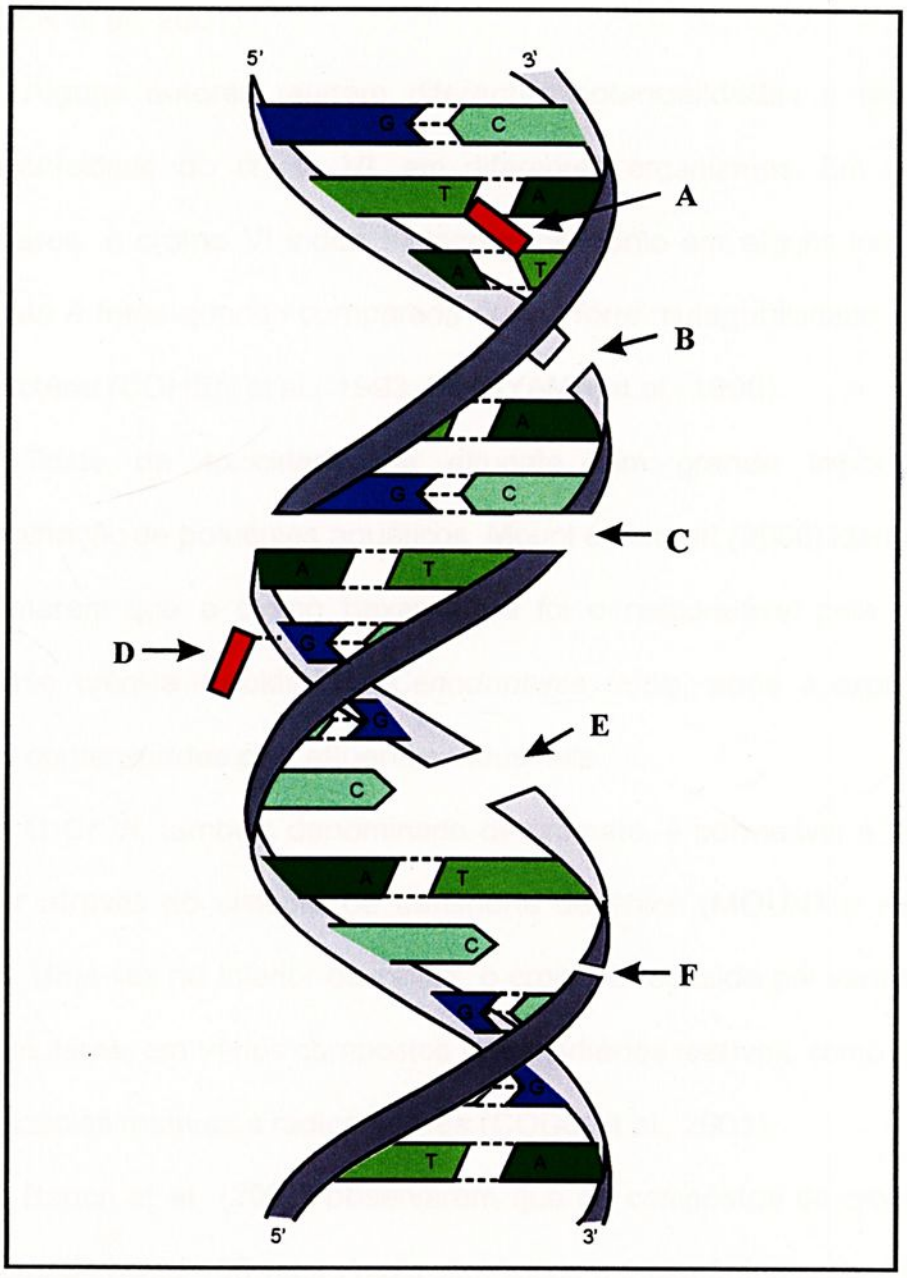

Figura 3 - Representação esquemática de danos do DNA, promovidos por agentes mutagênicos. **A-** Adutos de DNA ligados a nucleotídeos (ligação aduto-timinas). **B-** Quebra de fita simples; **C-** Quebra de fita dupla; **D-** Adutos de DNA ligados ao fosfato da dupla hélice pela ação de aminoácido (cisteína, histidina, ácido glutâmico e glutationa); **E-** Excisão de nucleotídeo; **F-** Excisão de base (depurinação da guanina).

cromátides irmãs e erros na síntese de DNA (ZHITKOVICH et al., 1996; O'BRIEN et al., 2001).

Alguns autores relatam diferentes potencialidades à resposta de mutagenicidade do cromo VI, em diferentes organismos. Em células de mamíferos, o cromo VI induz mutações de ponto em alguns locos, mas a resposta é fraca quando comparada com a forte mutagenicidade observada em bactéria (COHEN et al., 1993; SUGIYAMA et al., 1996).

Teste de toxicidade de efluente tem grande importância na determinação de poluentes aquáticos. Mount e Hockett (2000) identificaram e confirmaram que o cromo hexavalente foi o responsável pela toxicidade aguda e crônica exibida por *Ceriodaphinia dúbia*, após a exposição em águas contaminadas com efluentes industriais.

O Cr VI, também denominado de cromato, é permeável à membrana celular através do sistema de transporte de ânion (MOUNT e HOCKETT, 2000). Uma vez no interior da célula, o cromo é reduzido por várias reações intracelulares, em vários compostos intermediários reativos, como o Cr V, Cr II e espécies reativas a radicais livres (CODD et al., 2001).

Bagch et al. (2002) observaram que os compostos de cromo VI são aproximadamente 100 vezes mais citotóxicos e mutagênicos que o composto de cromo III, quando testados em culturas de fibroblasto humano.

O composto de Cr VI é pouco reativo, *in vitro*, com a molécula de DNA e com as proteínas. Já o composto de Cr III se liga fortemente com o DNA e com as proteínas, em testes *in vitro* (CODD et al., 2001) (Figura 3).

Os cromatos insolúveis (Cromo III) são absorvidos pela célula via fagocitose, cujas vesículas são vacúolos ácidos pH 3-5 (SINGH et al., 1998) (Figura 2).

A teoria de que o Cr VI é transportado para o interior da célula através dos canais de transferência de ânions, endógenos isoestruturais e isoeletrônicos, como SO_4^{-2} e HPO_4^{-2}, foi confirmada por Beyersmann et al., (1994) (Figura 2). Os autores relatam que a quantidade de Cr VI *uptake* foi reduzida, significativamente, na presença de reagentes conhecidos como bloqueadores de canais transportadores de ânion.

O Cr III normalmente é incapaz de se difundir através da membrana celular (LEONARD e LAUWERYS, 1980). O composto de Cr III, existente no interior celular, geralmente é derivado da redução do Cr VI ou derivado de endocitose mediada por receptores. O cromo III dentro da célula apresenta uma alta ação mutagênica (WARREN et al., 1981; SUGDEN et al., 1990; SINGH et al., 1998). O Cr III é facilmente acumulado na superfície da célula, quando comparado com o Cromo VI (DEBETTO e LUCIANI, 1988). Esse acúmulo pode ser explicado pela variedade de sítios de ligação cátion-iônica na membrana celular, e à dificuldade no processo de transporte do Cr III para o interior da célula, que não é observado para o Cr VI (Figura 2).

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Como já citado, para que o Cr III seja disponibilizado para a célula, é necessário que o cromo VI seja reduzido, transformando-se assim em cromo III. A ausência de barreira celular para a entrada de cromo VI pode disponibilizar o Cr III, que é altamente mutagênico, por comprometer a fidelidade do mecanismo de replicação do DNA (CODD et al., 2001) (Figura 2).

A redução do Cr VI é feita por citocromo microssomal P-450, redutores (cisteína, glutationa e NADH) (CONNETT e WETTERHAHN, 1983; SINGH et al., 1998) e por enzimas mitocondriais (CONNETT e WETTERHAHN, 1985; SNOW, 1992).

O'Brien et al. (2001) confirmam que a glutationa (GSH) tem a capacidade de reduzir o Cr VI para Cr III, *in vitro*. Essa redução pode ocorrer sem a presença de um cofator ou de uma enzima. De acordo com os autores, essa reação é fortemente dependente do pH, sendo mais rápida em pH abaixo de 5.

De acordo com Borges et al. (1991) e O'Brien et al. (2001) o Cr VI intracelular, na presença de glutationa, produz uma espécie de Cr V que provoca alterações no DNA, tais como quebras, adutos de DNA-Cr, *crosslink* DNA-DNA, *crosslink* DNA-proteína, sítios apúricos/apirimídicos, e alterações oxidativas (Figura 3).

Sugden et al. (1990) verificaram que, nas reações de baixa concentração de Cr III (0.5 – 5.0 μM), a taxa de replicação é aumentada e a

fidelidade de replicação do DNA é diminuída, havendo conseqüentemente, uma indução do aumento dos danos na molécula desse DNA. Já as reações com alta concentração de Cr III, acima de 10μM, a taxa de replicação é baixa, pois o Cr III induz a formação de *crosslink* DNA-DNA.

O Cr III inibe a atividade da enzima envolvida na biossíntese de "pools" de nucleotídeos. Esse desbalanço compromete a fidelidade da replicação do DNA, provocando mutações nas células afetadas (ROJAS et al., 1999).

A ação do composto de cromo pode ser inibida por dietilmalimide (DEM) e agente bloqueador. O DEM pode bloquear a rápida redução intracelular do Cr hexavalente para trivalente, pela inibição da glutationa e/ou pela inibição de outro redutor intracelular (DEBETTO e LUCIANI, 1988).

O Cr VI interfere na atividade das enzimas e/ou no substrato envolvido na síntese de ATP que, provavelmente, ocorre durante o processo de formação do complexo de Cr III. Existe uma teoria de que a espécie de Cr V é formada pela redução do Cr VI por microssomo, na presença de NADPH, ou por glutationa na solução neutra (O'BRIEN et al., 2001).

Codd et al. (2001), trabalhando com células de cultura, relatam que o DNA isolado das reações do Cr V com glutationa ou do Cr VI com ácido ascórbico na presença de O_2, apresentou danos na sua estrutura. Estudos *in vitro* com Cr VI + redutor + DNA + O_2 têm sido realizados por O'Brien e Kortenkamp (1995) e Kortenkamp et al. (1996). A principal conclusão desses

estudos é que muitas espécies reativas, bem como os radicais livres resultantes do complexo de Cr V e IV, na presença de um redutor, formam o O_2 ligante, que altera o DNA igualmente ao observado em reação com radicais livres.

Kawanishi et al. (1986) demonstraram que o Cr V não é potencialmente carcinogênico. De acordo com os autores, a carcinogenicidade é dada pela formação de radical livre (ânion peróxido e radical hidroxila), formado durante a reação de redução do Cr VI. Essas ROS são eficientes na promoção de alteração da estrutura do DNA.

Jones et al. (1991) sugerem que os radicais hidroxila (•OH), gerados do Cr V são responsáveis pela quebra da molécula de DNA.

$$Cr\ III + O_2\bullet^- \rightarrow Cr\ II + O_2$$

$$Cr\ II + H_2O_2 \rightarrow Cr\ I + \bullet OH + OH^-$$

$$Cr\ VI + O_2\bullet^- \rightarrow Cr\ V + O_2$$

$$Cr\ V + H_2O_2 \rightarrow Cr\ VI + \bullet OH + OH^-$$

Etapas de redução do Cromo, gerando os radicais livres.

Ozawa e Hanaki (1990) defendem que o Cr III é relativamente não tóxico, quando reduzido para Cr II por redutores biológicos, como L-cysteína

NADH. Esse Cr II reage com o peróxido de hidrogênio para produzir radicais hidroxilas, que são responsáveis pelos danos nos tecidos.

A reatividade dos aminoácidos para a formação do complexo DNA-Cr III foi examinado, *in vitro,* com diferentes tipos de aminoácidos ativos. Os aminoácidos cisteína, histidina, tirosina, metionina e treonina foram os considerados mais ativos, quanto à potencialidade de ligação com o complexo cromato com o DNA (Zhitkovich et al., 1996).

A cisteína e a histidina, como vários outros aminoácidos que contêm hidroxil, tiveram sítios da proteína muito reativos com o Cr III. Esses estudos sugerem que o Cr III, de fato, interage e se liga à molécula de DNA, *in vitro,* e que o Cr VI pode ser reduzido, *in vitro,* para a forma Cr III (CODD et al., 2001).

Estudos *in vitro* mostram que Cr III se liga à molécula de DNA, preferencialmente, através de certas bases. Essa interação preferencial foi baseada nos resultados da análise da ligação do Cr III com o polirribonucleotídeo e não do cromo especificamente com o DNA. A ligação do cromo III com o polirribonucleotídeo G provavelmente não se deu, especificamente, pela ligação com uma base, mas pela estrutura *tetrastrand* de polirribonucleotídeo, que pode favorecer a ligação do Cr III a um sítio fosfato específico, presente na *tetrastrand* (CODD et al., 2001). A interação entre Cr III – histidina-nucleosídeo sugere, que o grupo fosfato é o primeiro sítio de ligação do DNA, para o complexo Cr-aa.

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Segundo Codd et al. (2001), o Cr III estimula a formação dos complexos aminoácido-DNA e proteína-DNA. A atividade do complexo DNA-proteína é dependente da quantidade de aminoácido reativo.

Voitkun et al. (1994) observaram que as células tratadas com chumbo cromado formaram adutos Cr-DNA pelo envolvimento com aminoácidos ligantes. A cisteína, histidina, ácido glutâmico e glutationa são os principais ligantes responsáveis pela ligação do Cr III ao DNA.

Zhitkovich et al. (1996) analisaram o mecanismo de formação do complexo aminoácido-Cr III-DNA, *in vitro,* em que se evidenciou a importância do grupo fosfato do DNA como o sítio de ligação do composto de Cr VI e III. Foi observada também uma ausência de especificidade de bases no Cr-dependente de aminoácido-DNA. A formação de aminoácido (cisteína/histidina) – Cr III-DNA por Cr VI, sugere que o produto final do metabolismo do Cr VI, o Cr III, foi o responsável pela formação de aduto aminoácido-DNA. Os autores não encontraram formas intermediárias nos produtos dos adutos, observadas em dois importantes redutores biológicos: ácido ascórbico e cisteína.

A eficiência da ligação da cisteína com o Cr VI e Cr III, para oligonucleotídeo de composição base específica, reforça que o fosfato é o principal ponto de ligação entre os compostos do complexo (ZHITKOVICH et al., 1996). Porém, alguns autores, como Krishnamoorthy e Harris (1980), relatam que a cisteína pode não exibir interação com o Cr III.

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Há evidências de que o Cr V é a forma reativa de ligação do cromo com o DNA (Cr-DNA aduto). Os compostos de Cr V, gerados na presença de NADH, geralmente não se mostram reativos às bases de DNA ou aos mononucleotídeos (MOLYNEUX e DAVIES, 1995).

Experimentos de replicação do DNA, *in vitro*, realizados por Tkeshelashvili et al. (1980) e Schaaper et al. (1987), em polinucleotídeos sintéticos e/ou DNA do fago ∅X174, mostraram haver uma correlação direta entre presença do cromo III e incorporação incorreta de nucleotídeo.

Para a compreensão da possível formação do cromo III na indução da carcinogênese, Snow e Xu (1991) e Snow (1992) investigaram, *in vitro*, a ligação do Cr III com o DNA e o mecanismo de alteração induzida pelo Cr III na atividade da DNA polimerase, *in vivo*. Foi utilizado o DNA do bacteriófago M13 mp2 para a avaliação dos efeitos genéticos causados pela interação do Cr III com os complexos polimerase-molde e DNA-DNA. Os resultados desse experimento indicam que o $CrCl_3$ interfere na ação da enzima DNA polimerase, havendo um aumento da mutagenicidade.

O aumento da infidelidade da replicação do DNA produz danos oxidativos decorrentes da reação de redução do cromo, intracelularmente.

Para Okada et al. (1981), o Cr III tem alta influência na regulação da transcrição gênica. Segundo Snow e Xu (1991), a baixa concentração de $CrCl_3$ pode aumentar a síntese por poli I-K dentro e fora da seqüência *hairpin*

de M13mp7, podendo também aumentar a função da extensão do *primer* da polimerase II e pol. β, em eucarioto.

O Cr III pode aumentar a transcrição não-específica, pela RNA polimerase, *in vitro* (OKADA et al., 1981, 1983; OHBA et al., 1986). Snow e Xu (1991) sugerem que a incorporação de $CrCl_3$ tem a habilidade de aumentar a força de ligação da polimerase com o molde de DNA, havendo assim um aumento da taxa de incorporação de nucleotídeos durante cada ciclo de transcrição.

As altas concentrações de Cr III são conhecidas por ligarem preferencialmente ao DNA rico em guanina (TSAPAKOS e WETTERHAHN, 1983; WOLF e KASEMANN, 1989) ou em outros casos, ao não-pareamento direto ou a oxidação do promotor (AIYAR et al., 1989), ou ainda pela promoção de depurinação (SCHAAPER et al., 1987) da guanina, causando, subseqüentemente, um pareamento incorreto, modificando assim a base, caracterizando seu efeito mutagênico.

A associação comprovada do cromo com o DNA rico em guanina pode sugerir que o cromo tenha esse alto efeito mutagênico devido à alta relação entre DNA rico em guanina com eucromatina. Dessa forma, o efeito do cromo em porções ricas em guanina pode também refletir-se em um efeito direto na eucromatina, causando mutação em seqüências gênicas do DNA.

1.4 – Padrões de qualidade de água para a saúde pública e classificação das águas

De acordo com a origem, características, componentes, destino e uso das águas de ambientes lóticos, foram estabelecidos padrões para a sua classificação. A resolução nº 20 do CONAMA (Conselho Nacional do Meio Ambiente) de 18 de junho de 1986, estabeleceu, no estado de São Paulo, critérios de classificação das águas doces, salobras e salinas:

Classe Especial: Águas destinadas ao abastecimento doméstico em estado natural, sem prévia ou simples desinfecção, preservando o equilíbrio nas comunidades aquáticas, com restrições à presença de coliformes e qualquer outro tipo de rejeitos;

Classe 1: destinadas ao abastecimento doméstico após tratamento simplificado de desinfecção, à proteção das comunidades aquáticas, à recreação de contato primário, à irrigação de hortaliças e de frutas consumidas cruas e cultivadas rente ao solo e à aqüicultura de espécies destinadas à alimentação humana.

Águas desta classe devem apresentar um padrão de qualidade que restringe substância lipídica, microorganismo patogênico, como os coliformes, substância orgânica e inorgânica, cancerígena ou tóxica, como metal pesado, biocida, organoclorado e fosforado, carbamatos, além de

substância que elimine odor, forme depósitos e flutue. Esses padrões também são estendidos às águas de classes 2 e 3;

Classe 2: Águas destinadas ao abastecimento doméstico, após tratamento convencional, à proteção das comunidades aquáticas, à recreação de contato primário (natação, esqui-aquático e mergulho), à irrigação de hortaliças e plantas frutíferas e à aqüicultura;

Classe 3: Águas destinadas ao abastecimento doméstico, após tratamento convencional, à irrigação de culturas arbóreas, cerealíferas e forrageiras, e à dessedentação de animais, usos esses menos exigentes em relação a sua potabilidade;

Classe 4: destinadas à navegação, à harmonia paisagística e aos usos menos exigentes, com padrão de qualidade que restringe apenas os materiais sedimentáveis, flutuantes e algumas substâncias químicas;

Classe 5 e 6: Águas salinas (alto teor de cloreto de sódio – marinha);

Classe 7 e 8: Águas salobras (acentuado teor de sais como bicarbonatos, cloretos, nitratos de cálcio e magnésio), sendo diferenciadas pelo tipo de uso à que se destinam.

Segundo o comitê das Bacias Hidrográficas, na situação atual, o rio Sapucaizinho encontra-se como rio de Classe 2, e o Córrego dos Bagres como de classe 4.

Os Municípios de Franca/SP e Patrocínio Paulista/SP constituem uma região com intensa atividade industrial referente a processamento de couro

(curtumes). Durante o curtimento do couro do boi são utilizadas várias substâncias tóxicas, consideradas nocivas ao meio ambiente. Nessa região, toda a água utilizada no curtume, antes de ser despejada no rio, passa por tratamento, na tentativa de reduzir os impactos da poluição, seguindo as determinações da Lei Estadual Título II, do Decreto nº 8468, de 8 de setembro de 1976.

2. – OBJETIVO

Frente ao quadro analítico exposto, o presente trabalho tem como objetivo investigar a possível presença de agentes contaminantes, com potencial citotóxico, genotóxico e clastogênico, que são despejados no Rio Sapucaizinho, Município de Patrocínio Paulista/SP e Córrego dos Bagres, Município de Franca/SP, por meio da análise da divisão celular de *Allium cepa*, sistema de micronúcleo em *Allium cepa* e *Oreochromis niloticus,* e ensaio do cometa em *Oreochromis niloticus* e células de CHO-K1.

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3. – ARTIGO 1

TOXIC AND GENETOXIC EFFECTS OF HEAVY METAL, EXCLUSIVELLY OF THE TRIVALENT AND HEXAVALENT CHROMIUM – A REVIEWS.

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ABSTRACT:

During the last years, the emission of heavy metals to the environment has increased, causing a severe negative impact to the ecosystems and seriously compromising human health due to their mutagenic potential. Tri- (III) and hexavalent (VI) chromium (Cr) constitute the oxidative states of the metal chromium that are active in living organisms. These two oxidation states of the chromium differ with regards to their cellular effects, mainly due to the different abilities they possess in relation to easy of transport through biological membranes. Cr VI is transported into the cell through transference channels of endogenous anions that are isostructural and isoelectronical to Cr VI, such as SO_4^{-2} and HPO_4^{-2}. On the other hand, Cr III is unable to diffuse through the cell membrane. Its existence inside the cells is generally due to the reduction of Cr VI, the endocytosis, or the absortion by the cells via phagocytosis. Cr III acts directly on the DNA molecule, while Cr VI is less reactive to this molecule. Nevertheless, Cr VI is more dangerous to ecosystems since it is easily incorporated into the cell through the cell membrane. Once inside the cell system, it is bioreduced into Cr III, which is a potential mutagen. For this reason, several studies *in vivo* and *in vitro* have shown that Cr VI damages the DNA molecule in several ways, including breakage to the main strand and oxidation of purines and pyrimidines, which are alterations that generate chromosomal aberrations, micronucleus

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formation (MN), exchanges between sister chromatids, and errors in DNA replication.

Key Words: Chromium, genotoxic, heavy metal, changes DNA

INTRODUCTION

Mutagens are substances that induce changes in the DNA molecule that can be corrected by the cell repair mechanism of the cells themselves. Unrepaired or erroneously repaired changes give origin to point and/or chromosomal mutations (Connor and Fergunson, 1993).

Most chemical substances with a genotoxic potential can alter the genetic material in all phases of the cell cycle; however, to become fixed as chromosomal changes, they require a mechanism for DNA replication which must be activated immediately after the occurrence of these alterations (Obe et al. 1982). Many mutagens have effects on euchromatic nuclei that can be detected cytologically by the inhibition and interruption of the cell division process, by numerical and structural chromosome aberrations, sister chromatid exchanges, and other events.

Investigations of the action of environmental mutagens are acquiring increasing importance because, even though spontaneous mutations occur, most mutations are known to be induced by physical, chemical or biological agents to which man may be exposed.

Heavy metals may be present in several environments. Their presence in air, water, soil and foodstuffs may be harmful to health by inducing harmful changes in genetic material (Pascalicchio, 2002).

Biochemical analyses carried out on blood, urine and hair can provide reliable data for the determination of the magnitude of problems derived from

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environmental contamination with heavy metals. A hair exam, for example, permits the analysis of the variations in the concentrations of toxic metals along a given period of time (Pascalicchio, 2002).

The heavy metal contaminations more commonly observed in the environment are those caused by aluminum, arsenium, barium, cadmium, lead, chromium, mercury, and nickel.

Heavy metals have several mechanisms of action and can cause serious damage to cells. The mechanisms of action more commonly observed are changes in immunosuppressive activity, competition with enzyme activity co-factors at specific sites, inhibition of vital enzymes such as those of oxidative phosphorylation, and changes in cell structure, especially in the lipoprotein portion of the membrane (Pascalicchio, 2002).

GENOTOXICITY OF HEAVY METALS

All living organisms interact with the environment. Thus, their genome is exposed to the interferences suffered by this environment. Interaction between the environment and the organism results in changes that, when positive, may reflect adaptation of the organism to a better exploitation of this environment, due to the modification of the environment itself by the organism (Minissi and Lombi, 1997; Pascalicchio, 2002).

Over the last few years, the emission of heavy metals into the environment has increased, with a severe impact on ecosystems and on human health (Snow, 1992; Würgler and Kramers, 1992; Majer et al., 2002).

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Industrial, agricultural and urban activities are the major activities responsible for soil and water contamination with heavy metals, resulting in serious environmental problems (Briat and Lebrun, 1999).

Heavy metals are potentially mutagenic and are seriously involved in environmental pollution. Some heavy metals have been proved to favor tumor formation in experimental organisms and in eventually exposed human beings (Sunderman, 1984; Friberg et al., 1985).

Se, Cd, Cr and Ni are metals normally occurring in small amounts in foodstuffs. However, when they occur in excess they are considered to be carcinogenic and/or mutagenic (Minissi and Lombi, 1997).

Metal derivatives and their metabolites induce changes in chromosome structure both *in vivo* and *in vitro*, inducing point mutations in prokaryotes and eukaryotes (Pascalicchio, 2002)

All organisms are commonly exposed to oxidant agents. These agents, when present in excessive amounts, may cause damage to biological molecules such as lipids, proteins, carbohydrates, and DNA (Sies, 1993; Halliwell et al., 1995). Heavy metals may be related to the production of these oxidants (Stohs and Bagchi, 1995).

Heavy metal compounds have been shown to be carcinogenic, possibly affecting the development and growth of some animals (De et al., 1990; Sunderman et al., 1995). The biotransformation of metals can be explained by interference with their normal function, with the ability to interact

with specific sites of essential elements, impairing basal cell metabolism and consequently the metabolism of the organism as a whole.

According to Beyersmann et al. (1994), the heavy metal cadmium acts on cell metabolism related to the alteration in the balance between cell differentiation and proliferation. Cadmium has shown effects at various levels of cell transduction such as plasma membrane signals, increased free intracellular calcium which interferes with the metabolism of cell vesicle ATPase, activation and translocation of proteins that may activate tumor proliferation, and activation of oncogenic expression by the induction of oncogene transcription.

During cell metabolism, reduced oxygen forms are produced which may be catalyzed by heavy metals, generating free radicals that cause changes in macromolecules, contributing to the event of cell death (Briat and Lebrun, 1999).

Oxidant compounds such as hydrogen peroxides (H_2O_2), superoxides ($O_2\bullet$-) and hydroxyl radicals (OH•-) are classified as reactive oxygen species (ROS). With the exception of H_2O_2, ROS are considered to be free radicals by presenting an unpaired electron in their atomic structures (Haliweel and Gutteridge, 1985). The reduction of one electron in the oxygen molecule for the formation of a superoxide radical ($O_2\bullet$-) is thermodynamically unknown.

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Heavy metals in transition frequently present an unpaired electron, thus acquiring the property of being good catalyzers of O_2 reduction.

$$METAL^{n} + O_2 \rightarrow METAL^{n+1} + O_2\bullet^-$$

^{n} = umpaired electron
$^{n+1}$ = paired electron

Reaction of 0_2 with a heavy metal in the formation of ROS

In an aqueous solution and at neutral pH, the $O_2\bullet$ - generated in the above reaction may react with H_2O_2 and produce a hydroxyl radical ($OH\bullet$-). This hydroxyl radical may damage biological molecules, ultimately leading to cell death (Briat and Lebrun, 1999). The reaction between H_2O_2 and O_2 is called Haber-Weiss reaction, as per the scheme below.

$$O_2\bullet^- + H_2O_2 \rightarrow O_2 + OH^- + OH\bullet$$

Haber – Weiss Reaction

When iron is the metal in transition in the Haber-Weiss reaction, the reaction is called Fenton reaction (Halliwell, 1990), as described below:

$$Fe\ III + O_2\bullet^- \rightarrow Fe\ II + O_2$$

$$Fe\ II + H_2O_2 \rightarrow Fe\ III + OH\bullet + OH^-$$

Fenton Reaction

Hydroxyl radicals can be produced close to the DNA molecule, adding to or removing from these DNA bases an H atom (Pryor, 1988). This reaction can be responsible for changes in the DNA bases of the cell of the order of 10^4 to 10^5 per day (Ames et al., 1991).

Metal ions that participate in the Haber-Weiss reaction also have an important function in the oxidative modification of free amino acids and proteins (Stadtman, 1993).

Heavy metals can bind to molecules arranged in the cell nucleus through chemical reactions that generate ROS and consequently can lead to changes in DNA bases, in binding between DNA and inter- and intramolecular proteins, rearrangements, and depurination (Briat and Lebrun, 1999).

The toxicity of a given metal to various tissues can be explained by its action when in transition. Metals can act as catalyzers in the oxidative deterioration of biological macromolecules, producing ROS and resulting in lipid peroxidation and DNA alteration (Stohs and Baghi, 1995). O_2 and metals in transition, especially iron, are involved in the lipid peroxidation of biological membranes rich in polysaccharides and fatty acids (Scholz et al., 1990).

Methylation of the metal by ROS in the proximity of DNA mainly generates the promutagen adduct 8-OXO-G (7,8-dihydro-8-oxoguanine). These adducts may pair with the A base, a fact that, in the absence of DNA

repair, will result in a mutation of the type of G to T base transduction (Cunningham, 1997), also called frameshift mutation (Figure 1).

Alleman et al. (1985) and Minotti and Aust (1987) reported the participation of Fe II and Fe III in the process of initiation of lipid peroxidation by ROS generation, demonstrating that Fe is a good catalyzer in the process of ROS formation. Iron can catalyze the reaction of reduction between oxygen and biological macromolecules. In the absence of Fe, this reduction does not occur.

ROS such as H_2O_2 and $O_2\bullet^-$ are generated *in vivo* in all aerobic cells. Under physiological conditions, these reactive species do not react with DNA, unless metal ions are present, permitting the formation of OH•, a species highly harmful to the organism (Blakely et al., 1990).

Reactions catalyzed by Fe and Cu ions seem to be more important *in vivo* in reactions with $O_2\bullet^-$.

Copper acts as a catalyzer in processes of ROS formation, in lipid membrane peroxidation (catalysis) (Chan et al., 1982) and in hydroquinone and benzoquinone oxidation (Li and Trush, 1993). Ozawa et al. (1993) reported that a Cu II complex can react with hydrogen peroxide to produce a high concentration of hydroxyl radicals which is directly responsible for DNA alterations. The reaction of hydroxyl radical formation using Cu as a catalyzer is as follows:

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$$Cu\ II + O_2\bullet^- \rightarrow Cu\ I + O_2$$

$$2\ O_2\bullet^- + 2\ H^+ \rightarrow H_2O_2 + O_2$$

$$Cu\ I + H_2O_2 \rightarrow Cu\ II + \bullet OH + OH^-$$

Formation of hydroxyl radical, using Cu as a catalyzer

Metal ions such as Fe III, Mn III, Cd II and Zn II are not significant accelerators of hydroquinone oxidation or of induction of breaks in the DNA strand (Li and Trush, 1993). However, Cu plays an important role in ROS generation, cytotoxicity, and induction of DNA damage by hydroquinone in target cells.

Studies by Robertson (1985) and L'Huillier et al. (1996) have shown that nickel accumulation in maize roots reduced the mitotic activity of the plant's meristems, probably due to the loss root meristem integrity. Chakravarty and Srivastava (1992), in a study on sunflowers, reported that, depending on concentration and time of exposure, aluminum, cadmium, copper and nickel increase the clastogenic effect in this plant.

GENOTOXICITY OF CHROMIUM (Cr)

Chromium compounds have toxic, genotoxic, mutagenic and carcinogenic effects on man and animals (Von Burg and Liu, 1993; Stohs and Bagchi, 1995; Mount and Hockett, 2000). According to Von Burg and Liu (1993), chromium has acute toxicity, chronic toxicity, neurotoxicity,

reproductive toxicity, genotoxicity, and carcinogenotoxicity, in addition to contaminating the environment.

Epidemiological studies have demonstrated that workers in the chromate industry are more predisposed to the development of lung cancer. A high rate of sinonasal cancer has been reported to occur among industrial workers exposed to primary chromate products in Japan and in the US (Rojas et al., 1999).

Trivalent chromium III and hexavalent VI chromium are in an active oxidative state in living beings. These two stages of chromium oxidation differ in terms of their cell activities, mainly due to the different abilities they have to cross biological membranes (Leonard and Lauwerys, 1980; Stohs and Bagchi, 1995). Chromium VI has a tetrahedral geometrical structure which is smaller and therefore crosses biological membranes more easily than chromium III, which has an octahedral geometrical structure (Figure 2).

Data reported by Debetto et al. (1982) have suggested that not only Cr III reacts with DNA, but that Cr VI may produce genotoxicity through an indirect mechanism, generating reactive oxygen intermediates. Several *in vivo* and *in vitro* studies have shown that Cr VI induces a variety of damaging effects on the DNA molecule, including breaks in the main chain and base oxidation, which generate chromosomal aberrations (Figure 3), micronucleus (MN) formation, sister chromatid exchanges, and errors in DNA synthesis (Zhitkovich et al., 1996; O'Brien et al., 2001).

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Some investigators have reported different potential responses to mutagenicity in different organisms exposed to chromium VI. In mammalian cells, chromium VI induces point mutations at some loci, but the response is weak compared to the strong mutagenicity observed in bacteria (Cohen et al., 1993; Sugiyama et al., 1996).

Mount and Hockett (2000) identified and confirmed the fact that hexavalent chromium is responsible for the acute and chronic toxicity occurring in *Ceriodaphinia dubia* after exposure to waters contaminated with industrial effluents.

Cr VI, also called chromate, can cross the cell membrane through the system of anion transport (Mount and Hockett, 2000). Once inside the cell, Cr is reduced by several intracellular reactions to various intermediate reactive compounds such as Cr V, Cr II and species reacting with free radicals (Codd et al., 2001).

Bagch et al. (2002) observed that Cr VI compounds are approximately 100 times more cytotoxic and mutagenic than Cr III compounds when tested on human fibroblast cultures.

The Cr VI compound is little reactive *in vitro* with the DNA molecule and with proteins, whereas the Cr III compound strongly binds to DNA and to the proteins in *in vitro* tests (Codd et al., 2001).

Insoluble chromates (Cr III) are absorbed by the cell by pinocytosis, with the vesicles being acid vacuoles, pH 3-5 (Singh et al., 1998) (Figure 2).

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The theory that Cr VI is transported into the cell through endogenous isostructural and isoelectronic anion transfer channels such as SO_4^{-2} and HPO_4^{-2} was confirmed by Beyersmann et al. (1994). The cited investigators reported that the amount of Cr VI uptake was significantly reduced in the presence of reagents known to be blockers of anion transport channels.

Cr III normally is unable to diffuse through the cell membrane (Leonard and Lawerys, 1980). The Cr III compound existing inside the cell usually derives from the reduction of Cr VI or from receptor-mediated endocytosis (Figure 2). Chromium III has a high mutagenic action within the cell (Warren et al., 1981; Sugden et al., 1990; Singh et al., 1998).

Cr III is easily accumulated on the cell surface compared to Cr VI (Debetto and Luciani, 1988). This accumulation may be explained by the variety of cationic binding sites on the cell membrane and by the difficulty in the process of Cr III transport into the cell, which is not observed for Cr VI (Figure 2).

As mentioned earlier, for Cr III to be made available to the cell, Cr VI must be reduced, thus being transformed to Cr III. The absence of a cell barrier against the entry of Cr VI can make available Cr III, which is highly mutagenic by compromising the fidelity of the mechanism of DNA replication (Codd et al., 2001) (Figure 2).

The reduction of Cr VI (Figure 2) is performed by cytochrome microsomal P-450, by reducing agents (cysteine, glutathione and NADH)

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(Connett and Wetterhahn, 1983; Singh et al., 1998), and by mitochondrial enzymes (Connett and Wetterhahn, 1985; Snow, 1992).

O'Brien et al. (2001) confirmed that glutathione (GSH) has the ability to reduce Cr VI to Cr III *in vitro*. This reduction can occur without the presence of a cofactor or an enzyme. According to the cited authors, this reaction strongly depends on pH and is more rapid at pH below 5.

According to Borges et al. (1991) and O'Brien et al. (2001), intracellular Cr VI, in the presence of GSH, produced a Cr V species that caused DNA changes such as breaks, DNA-Cr adducts, DNA-DNA crosslinks, DNA-protein crosslinks, apurine/apyrimidine sites, and oxidative alteration (Figure 3).

Sugden et al. (1990) observed that in reactions with low Cr III concentrations (0.5-5.0 μM) the rate of replication is increased and the fidelity of DNA replication is reduced, with a consequent induction of increased damage to the DNA molecule. In contrast, in reactions with a high Cr III concentration, above 10 μM, the rate of replication is low because Cr III induces the formation of a DNA-DNA crosslink.

Cr III inhibits the activity of the enzyme involved in the biosynthesis of nucleotide pools. This imbalance impairs the fidelity of DNA replication, provoking mutations in the affected cells (Rojas et al., 1999).

The action of chromium compounds can be inhibited by dielthylmalimide (DEM), an blocking agent. DEM can block the rapid intracellular reduction of hexavalent Cr to trivalent Cr by inhibition of

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glutathione and/or by inhibition of another intracellular reducing agent (Debetto and Luciani, 1988).

Cr VI interferes with the activity of the enzymes and/or substrate involved in ATP synthesis, an effect that probably occurs during the process of Cr III complex formation. There is a theory stating that that the Cr V species is formed by reduction of Cr VI by a microsome in the presence of NADPH, or by glutathione in a neutral solution (O'Brien et al., 2001).

Codd et al. (2001), working with cell cultures, reported that DNA isolated from the reactions of Cr V with glutathione or of Cr VI with an ascorbic system in the presence of O_2 presented structural damage. *In vitro* studies on Cr VI + reducing agent + DNA + O_2 have been conducted by O'Brien and Kortenkamp (1995) and Kortenkamp et al. (1996), who concluded that many reactive species, as well as the free radicals resulting from the Cr V and IV complex in the presence of a reducing agent, form binding O_2 which alters DNA in the same way as observed in reactions with free radicals.

Kawanishi et al. (1986) demonstrated that Cr V is not potentially carcinogenic. According to these authors, carcinogenicity is induced by the formation of free radicals (peroxide anion and hydroxyl radical) during the reaction of Cr VI reduction and theses ROS are efficient in causing changes in the structure of DNA.

Jones et al. (1991) have suggested that hydroxyl radicals (•OH) generated from Cr V are responsible for the break of the DNA molecule.

$$Cr\ III + O_2\bullet^- \rightarrow Cr\ II + O_2$$

$$Cr\ II + H_2O_2 \rightarrow Cr\ I + \bullet OH + OH^-$$

$$Cr\ VI + O_2\bullet^- \rightarrow Cr\ V + O_2$$

$$Cr\ V + H_2O_2 \rightarrow Cr\ VI + \bullet OH + OH^-$$

Stages of chromium reduction, gerating free radicals

Ozawa and Hanaki (1990) argue that Cr III is relatively nontoxic when reduced to Cr II by biological reducing agents such as L-cysteine and NADH. This Cr II reacts with hydrogen peroxide to produce hydroxyl radicals that are responsible for tissue damage.

Amino acid reactivity for the formation of the DNA-Cr III complex was examined *in vitro* with different types of active amino acids. Cysteine, histidine, tyrosine, methionine and threonine were considered to be the most active amino acids in terms of the potential for binding to the chromium-DNA complex (Zhitkovich et al., 1996).

Cysteine and histidine, like several other amino acids that contain a hydroxyl group, showed protein sites highly reactive to Cr III. These studies suggest that Cr III, in fact, reacts with and binds to the DNA molecule *in vitro* and that Cr VI can be reduced to the Cr III form *in vitro* (Codd, 2001).

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In vitro studies have shown that Cr III preferentially binds to the DNA molecule through certain bases. This preferential interaction was based on the results of analysis of Cr III binding with the polyribonucleotide and not of chromium specifically with DNA. Chromium III binding with the polyribonucleotide G probably did not occur specifically by binding to a base, but by the tetra-strand structure of the polyribonucleotide, which may favor Cr III binding to a specific phosphate site present in the tetra-strand (Codd, 2001). The Cr III-histidine-nucleoside interaction suggests that the phosphate group is the first DNA binding site for the Cr-aa complex.

According to Codd (2001), Cr III stimulates the formation of amino acid-DNA and protein-DNA complexes. The activity of the DNA-protein complex depends on the amount of reactive amino acids.

Voitkun et al. (1994) observed that cells treated with chromated lead formed Cr-DNA adducts by involvement with binding amino acids. Cysteine, histidine, glutamic acid and glutathione are the major ligands responsible for Cr III binding to DNA.

Zhitkovich et al. (1996) analyzed the mechanism of formation of the amino acid-Cr III-DNA complex *in vitro* and demonstrated the importance of the phosphate group of DNA as the binding site for the Cr VII and Cr III complex. They also observed the absence of base specificity in the amino acid-dependent-Cr-DNA complex. The formation of an amino acid (cysteine/histidine)-Cr III-DNA complex by Cr VI suggested that the final

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product of Cr VI metabolism, Cr III, was responsible for the formation of the amino acid-DNA adduct. The authors did not detect intermediate forms in the adduct products, which are observed in two important biological reducing agents, i.e. ascorbic acid and cysteine.

The efficiency of cysteine binding to Cr VI and Cr III for oligonucleotides of base-specific composition supports the fact that phosphate is the major binding site between the compounds of the complex (Zhitkovich et al., 1996). However, other investigators, such as Krishnamoorthy and Harris (1980) have reported that cysteine may not show interaction with Cr III.

There is evidence that Cr V is the reactive form of chromium binding to DNA (Cr-DNA adduct). The Cr V compounds generated in the presence of NADH usually are not reactive to DNA bases or to mononucleotides (Molyneux and Davies, 1995).

Experiments of *in vitro* DNA replication performed by Tkeshelashvili et al. (1980) and Schaaper et al. (1987) in synthetic polynucleotides and/or DNA of the ∅X174 phage showed that there is a direct correlation between the presence of chromium III and incorrect nucleotide incorporation.

To determine the possible formation of chromium III in the induction of carcinogenesis, Snow and Xu (1991) and Snow (1992) investigated Cr III binding to DNA *in vitro* and the mechanism of alteration induced by Cr III in DNA polymerase activity *in vivo*. DNA of the M13 mp2 bacteriophage was

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used to assess the genetic effects of the interaction of Cr III with the polymerase-template and DNA-DNA complexes. The results of their experiments indicated that $CrCl_3$ interferes with the action of the enzyme DNA polymerase, with the occurrence of increased mutagenicity.

An increase in the unreliability of DNA replication produces oxidative damage due to the intracellular reduction of chromium.

According to Okada et al. (1981), Cr III has a strong influence on the regulation of gene transcription. According to Snow and Xu (1991), a low concentration of $CrCl_3$ may increase poly I-K synthesis inside and outside the hairpin sequence of M13mp7, possibly also increasing the function of extension of the polymerase II and pol. β primer in eukaryotes.

Cr III may increase nonspecific transcription by RNA polymerase *in vitro* (Okada et al., 1981; Ohba et al., 1986). Snow and Xu (1991) have suggested that $CrCl_3$ incorporation has the ability to increase the strength of polymerase binding to the DNA template, with a consequent increase in the rate of nucleotide incorporation during each transcription cycle.

High Cr III concentrations are known to bind preferentially to guanine-rich DNA (Tsapakos and Wetterhahn, 1983; Wolf and Kasemann, 1989) or, in other cases, are known to be related to direct non-pairing or to promoter oxidation (Aiyar et al., 1989) or to promote guanine depurination (Schaaper et

al., 1987), subsequently causing incorrect pairing, thus modifying the base, an event that characterizes their mutagenic effect.

The confirmed association of chromium with guanine-rich DNA may suggest that chromium has this high mutagenic effect due to the high relationship between guanine-rich DNA and euchromatin. Thus, the effect of chromium on guanine-rich portions may also result in a direct effect on euchromatin, causing mutation in DNA gene sequences.

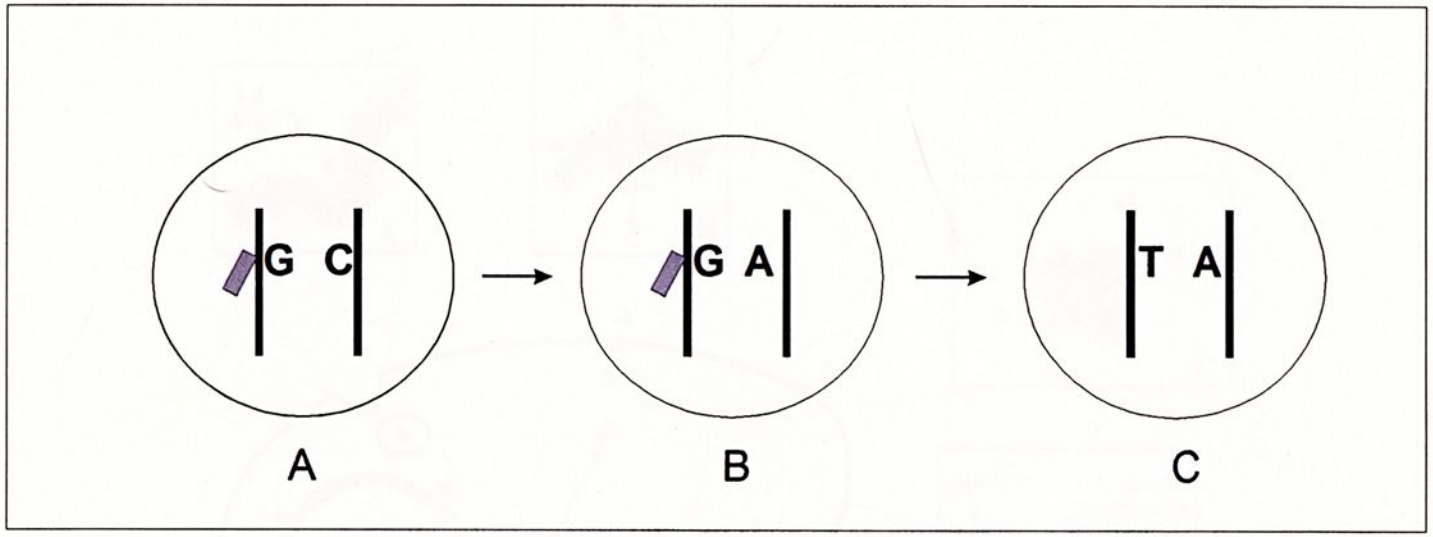

Figure 1 - **A**- adduct bound to the G base; **B**- pairing of A with G by the modified characteristic of G; **C** - new DNA synthesis with substitution of a base pair.

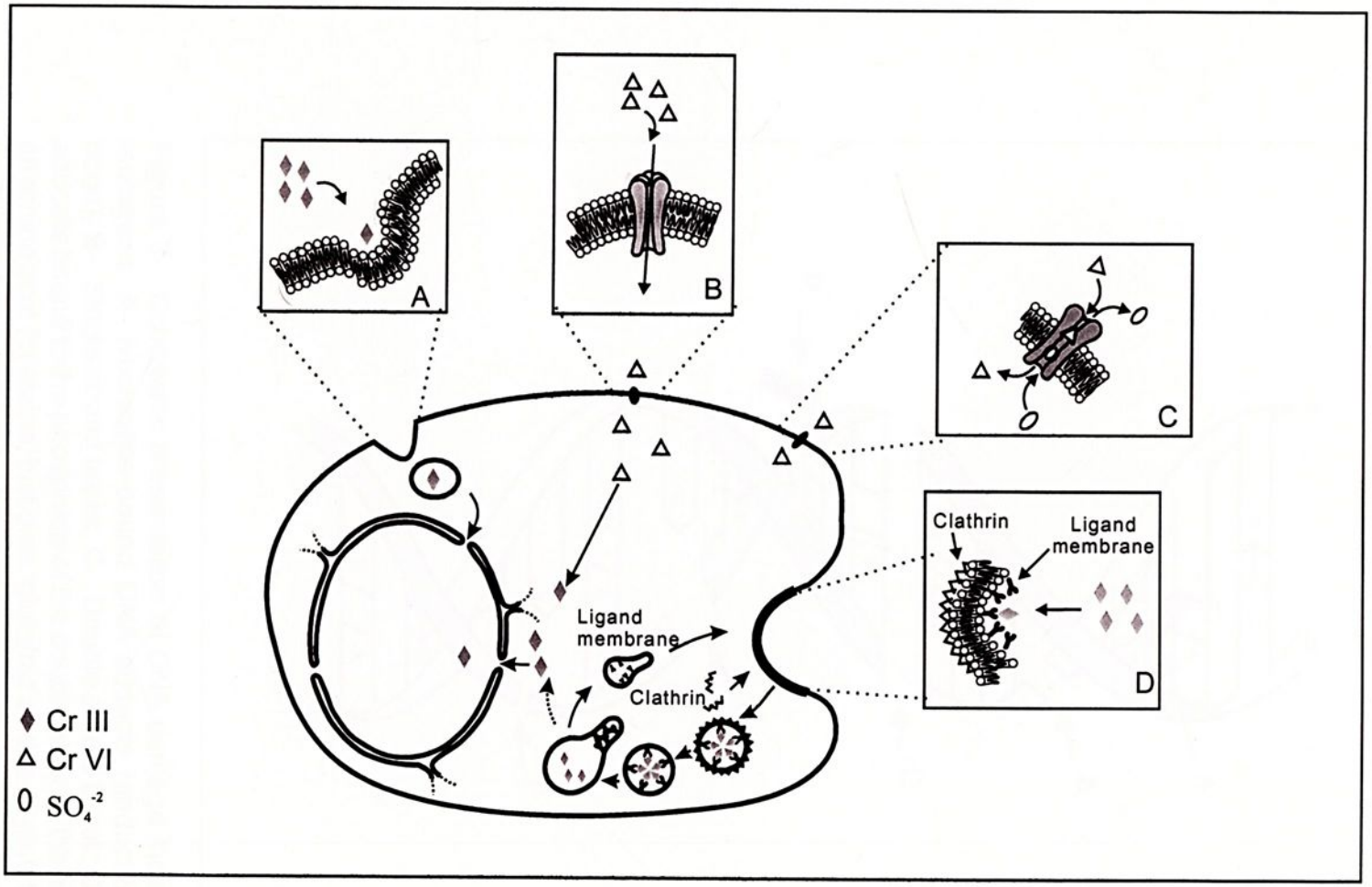

Figure 2 - Transmembrane transport pathways of chromium III and VI. **A**-Process of chromium III introduction into the cells (pinocytosis); **B**-Protein-mediated passive chromium VI transport; **C**-Active chromium VI transport mediated by an antiport-type protein; **D**- Receptor-mediated chromium III transport by endocytosis.

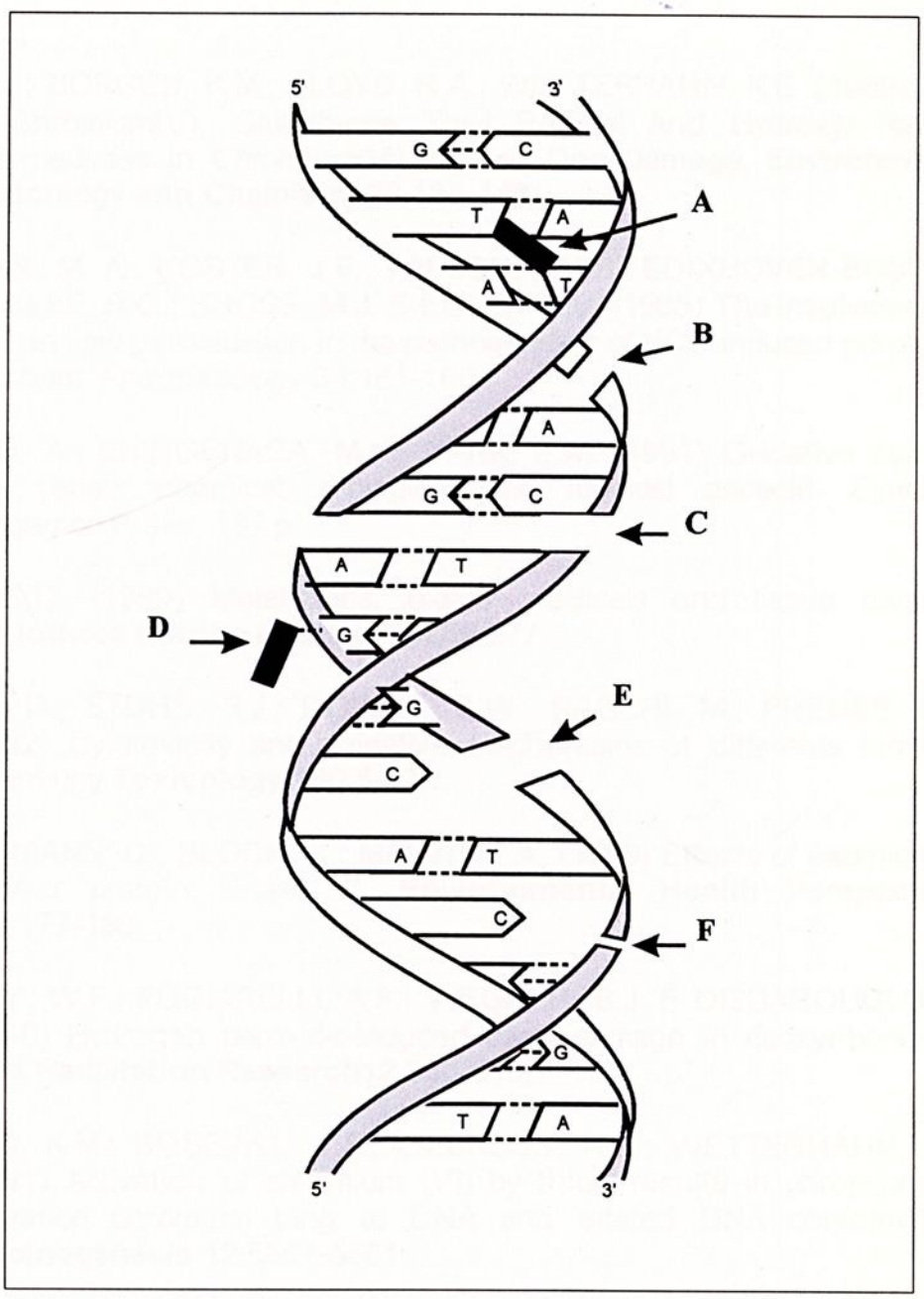

Figure 3- Schematic presentation of DNA damage induced by mutagens. **A-** Nucleotide-bound DNA adducts (adduct-thymine bond); **B-** Single strand break; **C-** Double strand break; **D-** DNA adducts bound to the phosphate of the double helix by the action of an amino acid (cysteine, histidine, glutamic acid, and glutathione); **E-** Nucleotide excision; **F-** Base excision (guanine depurination).

REFERENCE

AIYAR, J.; BORGES, K.M.; FLOYD, R.A.; WETTERHAHN, K.E. (1989) Role of Chromium(V), Glutathione Thiyl Radical And Hydroxyl Radical Intermediates In Chromium(Vi)-Induced Dna Damage. **Environmental Toxicology and Chemistry** 22:135-148.

ALLEMAN, M. A.; KOSTER, J.F.; WILSON, J.H.P.; EDIXHOVEN-BOSDIJK, A.; SLEE, R.G.; KROSS, M.J. E EIJK, H.G.V. (1985) The involvement of iron an lipid peroxidation in the pathogenesis of HCB-induced porphyria. Biochem. *Pharmacology* 34:161-166.

AMES, B. A.; SHINGENAGA, M.K.; PARK, E.M. (1991) Oxidative damage and repair: chemical, biological and medical aspects. Elmsford: Pergamon Press, 187 p.

AUST, S.D. (1989) Metal ions, oxygen radicals and tissue damage. **Bibliotheca nutritio et dieta** 43:266-277.

BAGCH, D.; STOHS, S.J.; DOWNS, B.W.; BAGCHI, M.; PREUSS, H.G. (2002) Cytotoxicity and oxidative mechanisms of differents forms of chromium. **Toxicology** 180:5-22.

BEYERSMANN, D.; BLOCK, C.; MALVIYA, A. (1989) Effects of cadmium on nuclear protein kinase C. **Envirronmental Health Perspectives** 102:177-180.

BLAKELY, W.F.; FUCIARELLI, A.F.; WEGHER, B.J. E DIZDAROUGLU, M. (1990) Hydrogen peroxide-induced base damage in deoxyribonucleic **Acid Radiatation Research**12:338-343.

BORGES, K.M.; BOSEWLL, J.S.; LIEBROSS, R.H.; WETTERHAHN, K.E. (1991) Activation of chromium (VI) by thiols results in chromium (V) formation chromium bing to DNA and altered DNA conformation. **Carcinogenesis** 12:5551-5561.

BRIAT, J.F.; LEBRUN, M. (1999) Plan responses to metal toxicity. **Sciences de la vie** 322:43-54.

CHAKRAVARTY, B.; SRIVASTAVA, S. (1992) Toxicity of some heavy metals *in vivo* and *in vitro* in *Helianthus annuus*. **Mutation Research** 283:287-294.

unesp

CHAN, P.C.; PELLER, O.G.; KESNER, L.(1982) Copper(II)-catalyzed lipid peroxidation in liposomes and erythrocyte membrane. **Lipids** 17:331-337.

CODD, R.; DILLON, C.T.; LEVINA, A.; LAY, P.A. (2001) Studies on the genotoxicity of chromium: from the test tube to the cell. **Coordination Chemistry Reviews** 216:537-582.

COHEN, M.D.; KARGACIN, B.; KLEIN, C.B.; COSTA, M. (1993) Mechanisms of chromium carcinogenicity and toxicity, **Critical riviews in toxicology** 23:255-281.

CONNETT, P.H.; WETTERHAHN, K.E. (1983) Metabolism of the carcinogen chromate by cellular-constituents. **Structure and bonding** 54:93-124.

CONNETT, P.H.; WETTERHAHN, K.E. (1985) *In vitro* Reaction Of The Carcinogen Chromate With Cellular Thiols And Carboxylic-Acids. **Journal of the american chemical society** 107:4282-4288.

CONNOR, J.A.; FERGUNSON, M.A. (1993) **Essential medical genetics**. Smith Blackwell Scientific Publications, 260p.

CUNNINGHAM, R.P. (1997) DNA repair: Caretakers of the genome? **Current biology** 7:576-579.

DE, S.K.; DEY, S.K.; ANDREWS, G.K. (1990) Cadmium teratogenicity and its relationship with metallothionein gene expression in midgestation mouse embryos. **Toxicology** 64:89-104.

DEBETTO, P.; TOSO, R.D.; VAROTTO, R.; BIANCHI, V.; LUCIANI, S. (1982) Effects of potassium dichromate on atp content of mammalian-cells cultured in vitro. **Chemico-biological interactions** 41:15-24.

DEBETTO, P.; LUCIANI S. (1988) Toxic Effect Of Chromium On Cellular-*Metabolism Science Of The Total Environmental* 71:365-377.

FRIBERG, L.; NORDBERG, G.F.; VOUK, V.B. (1985) **Handbook on the Toxicology of Metals.** 2nd ed., Amsterdam.

HALLIWELL, B.; GUTTERIDGE, J.M.C. (1985) Oxygen radicals and the nervous system. **Trends neurosciences** 8:22-26.

HALLIWELL, B. (1990) How to characterize a biological antioxidant. **Free radical research communications** 9:1-32.

HALLIWELL, B.; AESCHBACH, R.; LOLIGER, J.; AUOMA, O.I.(1995) The chracterization of antioxidants. **food and chemical toxicology** 33:601-617.

JONES, P.; KORTENKAMP, A.; O´BRIEN, P.; WANG, G.; YANG, G. (1991) Evidence for the generation of hydroxyl radicals from a chromium (V) intermediate isolated from the reaction of chromate with glutathione. **Arch. Biochemistry and Biophys** 286:652-655.

KAWANISHI, S.; INOUE, S.; SANO, S. (1986) Mechanism of DNA cleavage induced by sodium chromate (VI) in the presence of hydrogen peroxide. **Journal of biology chemistry** 261:5952-5958.

KRISHNAMOORTHY, C.R.; HARRIS, G.M. (1980) Equilibrium And Rate Study Of The Interaction Of Aqueous Chromium(III) Ion With Adenine. **Journal of coordination chemistry** 10:55-65.

KORTENKAMP, A.; CASADEVALL, M.; FAUX, S.P.; JENNER, A.; SHAYER, R.O.J.; WOODBRIDGE, N.; O´BRIEN, P. A (1996) role for molecular oxygen in the formation of DNA damage during the reduction of the carcinogen chromium(VI) by glutathione. **Arch. of biochemistry and biophysics** 329:199-207.

LEONARD, A.; LAUWERYS, R.R. (1980) Carcinogenicity and mutagenicity of chromium. **Mutation research** 76:227-239.

LI, Y.; TRUSH, M.A. (1993) Oxidation of hydroquinone by copper: Chemical mechanism and biological effects. **Arch. of biochemistry and biophysics** 300:346-355.

L´HUILLIER, L.; D´AUZAC, J.; DURAND, M.; MICHAUD-FERRIÈRE, N. (1996) Nickel effects on two maize (*Zea mays*) cultivars: growth, structure, Ni concentration, and localization. **Canadian journal of botany** 74:1547-1554.

MAJER, B.J.; TSCHERKO, D.; PASCHKE, A; WENNRICH, R.; KUNDI, M.; KANDELER, E.; KNASMÜLLER, S. (2002) Effects of heavy metal contamination of soils on micronucleus induction in *Tradescantia* and on

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microbial enzyme activities: a comparative investigation. **Mutation research** 515: 111-124.

MINISSI, S.; LOMBI, E. (1997) Heavy metal content and mutagenic activity, evaluated by *Vicia faba* micronucleus test, of Tiber river sediments. **Mutation research** 39:317-321.

MINOTTI, G.; AUST, S.D. (1987) The role of iron in the initiation of lipid peroxidation. **Chemistry and physics of lipids** 44:191-208.

MOLYNEUX, M.J.; DAVIES, M.J. (1995) Direct Evidence For Hydroxyl Radical-Induced Damage To Nucleic-Acids By Chromium(Vi)-Derived Species - Implications For Chromium Carcinogenesis. **Carcinogenesis** 16:875-882.

MOUNT, D.R.; HOCKETT, J.R. (2000) Use of toxicity identification evaluation methods to characterize, identify, and confirm hexavalent chromium toxicity in an industrial effluent. **Water research** 34:1379-1385.

OBE, G.; NATARAJAN, A.T.; PALITTI, F. (1982) Role of DNA doublestrand breaks in the formation of radiation – induced chromosomal aberrations. Progress in **Mutation research** 4:1-9.

O´BRIEN, P.; KORTENKAMP, A. (1995) The chemistry underlying chromate toxicity. **Transition metal chemistry** 20:636-642.

O'BRIEN, T.; XU, J.; PATIERNO, S.R. (2001) Effects of glutathione on chromium-induced DNA crosslinking and DNA polymerase arrest. **Molecular and cellular biochemistry** 222:173-182.

OHBA, H.; SUKETA, Y.; OKADA, S. (1986) Enhancement Of *In vitro* Ribonucleic-Acid Synthesis On Chromium(III)-Bound Chromatin. **Journal of inorganic biochemistry** 27:179-189.

OKADA, S.; OHBA, H.; TANIYAMA, M. (1981) Alterations In Ribonucleic-Acid Synthesis By Chromium(III). **Journal of inorganic biochemistry** 15:223-231.

OZAWA, T.; HANAKI, A. (1990) Spin-Trapping studies on the reactions of Cr(III) with hudrogen peroxide in the presence of biological reductants: Is Cr(III) nontoxic? **Biochemistry international** 22:343-352.

OZAWA, T.; UEDA, J.; SHIMAZU, Y. (1993) DNA single strand breakage by copper (II) complexes and hydrogen peroxide at physiological conditions. **Biochemistry and molecular biology international** 31:455-461.

PASCALICCHIO, A.E. (2002) **Contaminação por metais pesados**. São Paulo: Ed. Annablume, 132p.

PRYOR, W. A. (1988) Why is the hydroxyl radical the only radical that commonly adds to DNA? Hypothesis: it is a rare combination of high electrophiliciity, high thermochemical reactivity, and a mode of production that occurs near DNA. **Free radical biology and medicine** 4:219-223.

ROBERTSON, A.L. (1985) The poisoning of roots of *Zea mays* by nickel ions, and the protection afforded by magnesium and calcium. **New phytolologist** 100:173-189.

ROJAS, E.; HERRERA, L.A.; POIRIER, L.A.; OSTROSKY-EWGMAN, P. (1999) Are metals dietary carcinogens? **Mutation research** 443:157-181.

SCHAAPER, R.M.; KOPLITZ, R.M.; TKESHELASHVILI, L.K.; LOEB, L.A. (1987) Metal-iduced lethality and mutagenesis - possible role of apurinic intermediates. **Mutation research** 177:179-188.

SCHOLZ, R.W.; GRAHAM, K.S.; WYNN, M.K. (1990) Interaction of glutathione and α- tocapherol in the inhibition of lipid peroxidation of rat liver microsomes. In*:* REDDY, C.C.; HAMILTON, G.A. Biological Oxidation Systems, Ed. Madyastha, v.2. London: Academic Press, 867p.

SIES, H. (1993) Strategies Of Antioxidant Defense. **European journal of biochemistry** 215:213-219.

SINGH, J.; BRIDGEWATER, L.C.; PATIERNO, S.R. (1998) Differential sensitivity of chromium-mediated DNA interstrand crosslinks and DNA-protein crosslinks to disruption by alkali and EDTA. **Toxicological sciences** 45:72-76.

SNOW, E.T. (1992) Metal carcinogenesis: mechanistic implications. **Pharmacology and therapeutics** 53:31-65.

cm 1 2 3 4 5 6 7 unesp 12 13 14 15 16 17 18

SNOW, E.T.; XU, L. (1991) Chromium (III) bound to DNA templates promotes increased polymerase processivity and decreased fidelity during replication *in vitro*. **Biochemistry** 30:11238-11245.

STADTMAN, E.R. (1993) Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalysed reactions. **Annual review of biochemistry** 62:797-821.

STOHS, S.J.; BAGCHI, D. (1995) Oxidative mechanisms in the toxicity of metal ions. **Free radical biology and medicine** 18:321-336.

SUGDEN, K.D.; BURRIS, R.B.; ROGERS, S.J. (1990) An Oxygen Dependence In Chromium Mutagenesis. **Mutation research** 244:239-244.

SUGIYAMA, M.; TSUZUKI, K.; HARAMAKI, N. (1996) DNA single-strand breaks and cytotoxicity induced by sodium chromate (VI), **Mutation research** 369:3-21.

SUNDERMAN JR, F.W. (1984) Recent advances in metal carcinogenesis. **Annual clinic science** 14:93-122.

SUNDERMAN JR., F.W.; PLOWMAN, M.C.; KROFTOVA, S.; GRBAC-IVANKOVIC, S.; FOGLIA, L.; CRIVELLO, J.F. (1995) Effects of teratogenic exposure to Zn2+, Cd2+, Ni2+, Co2+ and Cu2+ on metallothionein content and metallothionein-mRNA contents of *Xenopus*embryos. **Pharmacology and toxicology** 76:178-184.

TKESHELASHVILI, L.K.; SHEARMAN, C.W.; ZAKOUR, R.A.; KOPLITZ, R.M.; LOEB, L.A. (1980) Effects Of Arsenic, Selenium, And Chromium on The Fidelity Of DNA-Synthesis. **Cancer research** 40:2455-2460.

TSAPAKOS, M.J.; WETTERHAHN, K.E. (1983) The Interaction Of Chromium With Nucleic-Acids. **Chemico-biological interactions** 46:265-277.

VOITKUN, V.; ZHITKOVICH, A.; COSTA, M. (1994) Complexing Of Amino-Acids To DNA By Chromate In Intact-Cells. **Environmental health perspectives** 102:251-255.

VON BURG, R.; LIU, D. (1993) Chromium and hexavalent chromium. **Journal of applied toxicology** 13:225-230.

WARREN, G.; SCHULTZ, P.; BANCROFT, D.; BENNETT, K.; ABBOTT, E.T.; ROGER, S. (1981) Mutagenicity of a series of hexacoordinate chromium (III) compounds. **Mutation research** 90:111-118.

WOLF, T.; KASEMANN, R. (1989) Ottenwalder, H. Molecular Interaction Of Different Chromium Species With Nucleotides and Nucleic-Acids. **Carcinogenesis** 10:655-659.

WÜRGLER, F.E.; KRAMERS, P.G. (1992) Environmental effects of genotoxins (eco-genotoxicology). **Mutagenesis** 7:321-327.

ZHITKOVICH, A.; VOITKUN, V.; COSTA, M. (1996) Formation of the amino acid-DNA complexes by hexavalent and trivalent chromium *in vitro*: Importance of trivalent chromium and the phosphate group. **Biochemistry** 35:7275-7282.

4. – ARTIGO 2

CYTOTOXIC AND GENOTOXIC EVALUATION OF THE WATERS OF A RIVER THAT RECEIVES TANNERY EFFLUENT USING THE *Allium cepa* TEST SYSTEM

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Papel submetted to Mutation research

Abstract

Several studies are being conducted to assess the toxicity and cytotoxicity of water bodies receiving industrial and domestic effluents, using the *A. cepa* test. To assess the toxicity and genotoxicity of waters possibly contaminated with chromium, derived from tannery activities, seasonal water collections were performed in 2001 and 2002 at five different sites along the Sapucaizinho river, Municiplaity of Patrocínio Paulista, State of São Paulo, Brazil. *Allium cepa* seeds were used as the test material and were submitted to germination in waters from the different collection sites, in Milli-Q water (negative control) and an aqueous solution of chromium (positive control). For the determination of cell division rates and mitotic irregularities, slides were prepared with root tip cells according to the standard Feulgen methodology. The results showed that the collection sites most heavily compromised by chromium emission presented low mitotic indices and a higher frequency of mitotic changes such as irregular anaphases (disorganized, multipolar, laggard), cells with chromosomal adherences, cells with micronuclei, and binucleate and/or multinucleate cells.

Keywords: Chromium; Clastogenicity; Genotoxicity; *Allium cepa*

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Introduction

The investigation of environmental agents with mutagenic potential has been the target of an increasing number of studies, since the consequences of their actions can result in mutations. The agents to which humans can be exposed are of a physical, chemical or biological nature.

Analysis of the genotoxic potential of a substance through the investigation of the induction of chromosome alterations represents an effective method for biomonitoring studies and for the analysis of the extent of pollution [1].

Many plant species have been used as a test organism in environmental monitoring assays. Some plant species such as *Allium cepa*, *Vicia faba* and *Tradescantia poludora* are efficient test organisms for the assessment of mutagenicity and clastogenicity resulting from the contamination with environmental pollutants [2,3].

Various studies have determined the toxicity and cytotoxicity of water resources that receive industrial and domestic effluents using the *A. cepa* test system [4, 5].

Analysis in water samples from the Paraguai river (Brazil) using the *A. cepa* test system, demonstrated the cytotoxic potential of these water samples at collection sites related to the emission of municipal wastewater [6]. In addition, the authors observed a seasonal influence on the cytotoxicity

of the samples analyzed, which was mainly related to seasonal cycles of flooding and drought.

Hexavalent (VI) chromium is a biologically active compound since the cell membrane is permeable to this metal. Once inside the cell, hexavalent chromium is rapidly reduced to trivalent chromium [7, 5]. *In vivo* and *in vitro* studies have shown that hexavalent chromium induces various types of damage to the DNA molecule, including chromosome aberrations, micronucleus formation, sister chromatid exchange, DNA strand breaks, and errors in the replication mechanism [8].

Trivalent (III) chromium in aqueous solution is unable to cross the cell membrane [9, 10], but can enter the cell through the processes of phagocytosis and endocytosis [5]. Once inside the cell, trivalent chromium binds to the DNA molecule and damages its structure, a process that characterizes its carcinogenic and mutagenic potential [11].

A. cepa roots was used as a test organism to determine the toxicity and genotoxicity of water possibly contaminated with hexavalent chromium in the State of Oressa (India) and observed diverse mitotic irregularities (C-mitosis, anaphase bridges, chromosome stickiness, and chromosome fragmentation and lagging), thus confirming the toxic and genotoxic effects of this metal [1,2]. Similar chromosome abnormalities were observed [13], when submitting *A. cepa* roots to different concentrations of trivalent and hexavalent chromium.

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Was determined the cytotoxic and genotoxic effect of chromium using *A. cepa* roots submitted to different concentrations of potassium dichromate and calcium chromate [1,4]. The authors observed an increase in the frequency of chromosome irregularities which was directly proportional to the chromium concentration in the solution. The most frequent chromosome abnormalities were anaphase aberrations, isochromosome formation, the presence of micronuclei, and centromere inactivation.

The Municipality of Patrocínio Paulista, São Paulo, is a region characterized by intense industrial activity related to leather processing (tanning). Various toxic substances including chromium, which are considered to be harmful to the environment, are used for the tanning of cow leather. In this region, all water used during tanning is submitted to treatment before being dumped into the river in an attempt to reduce the impact of pollution. The objective of the present study was to investigate the possible persistence of contaminating agents with mutagenic potential which are released during the tanning process into the Sapucaizinho River, Municipality of Patrocínio Paulista, by means of cytotoxicity and genotoxicity assays using *A. cepa* as the test organism.

Material and Methods

Seasonal collections were performed in 2001 and 2002 at five distinct sites along the Sapucaizinho River, Municipality of Patrocínio Paulista, State

of São Paulo, Brazil. The collection sites were standardized as follows: A – river water: 1 km before the disposal site of the tannery effluent (upstream of the river – PA), about 3 m before the disposal site (PB-A), at the disposal site (PB-L), and 1 km after the disposal site (downstream of the river – PC); B – previously treated tannery effluent water before disposal into the river (PB-T) (Figure 1).

The analyses were based on the Standard Methods for the Examination of Water and Wastewater [15].

For cation determination, the samples were first acidified with HNO_3, pH 1, and the cations were analyzed sequentially by inductively coupled plasma atomic emission spectrometry (ICP-AES) with ultrasonic nebulization. The following elements were determined: calcium, magnesium, strontium, silicon, iron, manganese, aluminum, zinc, chromium, cobalt, nickel, lead, cadmium, phosphorus, copper, and barium.

The standard solutions used to construct the calibration curves for the elements analyzed in 0.1% HNO_3 medium were obtained by appropriate dilution of the standard solution of 1000 ppm Titrisol (Merck).

A. cepa seeds were used as test material. Seeds were submitted to germination in water obtained from the different collection sites. Seeds submitted to germination in Milli-Q water only were used as negative control. For samples collected in 2002 an aqueous solution of trivalent chromium (0.089 mg/L) was used as positive control. The assays were performed with

seeds from a single *A. cepa* variety to avoid variations in the responses to the different steps of the procedure.

The seeds were submitted to two types of treatment: A) continuous treatment: soaking and germination of the seeds in the water derived from the collection sites; B) discontinuous treatment: the seeds were initially submitted to germination in Milli-Q water until reaching a length of 2 cm and then transferred to a Gerbox containing water from the collection sites and incubated for 20 h (acute treatment). After this period, some roots were randomly selected, while the remaining roots were maintained under the same conditions as described above until completing 72 h of treatment (chronic treatment) and then collected. For the assays performed with water samples collected in 2002, some roots were transferred to plates containing Milli-Q water after chronic treatment and incubated for a further 48 h (recovery treatment) before collection.

All root tips collected were fixed in Carnoy's solution diluted 3:1 (3 parts ethanol and 1 part acetic acid) for 24 h.

For cytological analysis, slides were obtained by the common method of gentle squashing. Root tips were submitted to acid hydrolysis in 1 N HCl at 60°C for 8 min, followed by washing in distilled water. The slides were stained with Schiff's staining for 2 h in the dark. All cells showing any type of alteration were recorded and the most significant cells were photographed for documentation of the material.

For the determination of the rate of cell division, slides containing root tip cells from both treatments were mounted as described above. Analysis was done taking into account the percentage of cells of each phase of cell division.

For assessment of the genotoxic potential, root tip cells showing irregular division such as irregular anaphases (disorganized, multipolar, delayed, etc.), cells with chromosome adhesions, cells with micronuclei, and binucleate and/or multinucleate cells were analyzed.

The results were analyzed statistically by the Kruskal-Wallis test.

Results and discussion

In the present study, *A. cepa* roots treated with water samples collected during the 2001 and 2002 rainy periods did not show significant differences in the mitotic index compared to the negative control for all treatments performed (Tables 1 and 2).

For water samples collected during the 2001 and 2002 dry periods, significant differences in the mitotic index were observed compared to the negative control for all treatments, except for the continuous treatment (Tables 1 and 2). In the case of the acute discontinuous treatment (20 h), the PB-T and PC sites showed mitotic indices close to that obtained for the positive control, with no significant difference (Tables 1 and 2). For the chronic discontinuous treatment (72 h), no significant difference in the mitotic

indices were observed between the PA, PB-T and PC sites and the positive control. These data agree with those reported [4, 6], whose results confirmed the potential of contaminating agents to reduce the mitotic index in *A. cepa*.

Analysis of the root tips submitted to the recovery treatment indicated a total reversal of the effects found for the acute and chronic treatments, since no significant differences were observed compared to the negative control. This efficient recovery of the mitotic index could also be observed for the treatment performed with trivalent chromium (positive control). These data suggest that the toxic effect of tannery effluents is indeed related to the presence of chromium, since recovery was observed both for samples obtained from the collection sites and for the positive control (Tables 1, 2, 3 and 4). These results confirm the toxic potential observed [13, 12] and indicate recovery from the cytotoxic effect in terms of the mitotic index altered by the action of chromium residues on the root tip cells of *A. cepa*. However, we suggest that, although the cells recover their division potential, they continue to present a cumulative mutagenic potential resulting from the action of chromium which persisted in subsequent generations, and, therefore, no recovery from these effects was observed (Tables 1 and 2).

A high frequency of aberrant cells was observed in *A. cepa* roots treated with water samples that receive tannery effluents (Tables 1 and 2). For samples collected in 2001, the frequency of aberrant cells in relation to the total number of dividing cells was higher at the PB-T site (water resulting

from tannery effluent treatment) during the dry period for the continuous (6.06) and acute discontinuous – 20 h (11.78) treatments (Table 1). As mentioned earlier, during the rainy period, the frequency of aberrant cells was low at all sites and for all treatments, with no significant differences compared to the negative control (Table 1). The most frequent aberrations identified in *A. cepa* root tip cells for the dry and rainy periods were cells with micronuclei and irregular anaphases (anaphase bridges and multipolar anaphases), as shown in Table 1 and Figure 2.

Analysis of *A. cepa* roots treated with water samples collected during the 2002 dry and rainy periods revealed a higher frequency of aberrations for the chronic discontinuous treatment (72 h). During the dry period, the highest frequency was observed at the PC site (downstream of the effluent), with a mean value of 9.62%, followed by the PB-T site (water resulting from tannery effluent treatment) with 9.23% and the PB-L site (site of effluent disposal) with 8.42%. These results indicate a genotoxic effect of the collected water samples probably caused by the chromium residues present in the tannery effluent and confirm by another reported [13, 12], who demonstrated the genotoxic potential of chromium present in water contaminated with this metal.

A high frequency of aberrant cells was observed during the 2002 rainy period for samples obtained from the PB-T site and submitted to continuous (9.33%) and chronic discontinuous (9.64%) treatments, for samples obtained

from the PB-A site and submitted to the acute discontinuous treatment (5.88%), and for samples obtained from the PC site and submitted to recovery treatment (8.23%).

Alterations probably induced by the action of chromium present in the water samples were observed during both the dry and rainy periods, with these changes occurring in both interphase and dividing cells. Abnormalities observed in interphase cells included cells with micronuclei and binucleate cells, with the former being the most frequent alteration. Mitotic irregularities included irregular anaphases (multipolar and anaphase bridges), chromosome breaks and losses, C-metaphases and chromosome adhesions (Table 2, Figures 2 and 3), with anaphase irregularities being the most frequent. These findings agree with other reported [13, 12] who described the potential of chromium to induce clastogenic effects (C-mitosis, anaphase bridges, and chromosome adhesions and fragments) in *A. cepa* roots.

Chromatid or chromosome breaks might be related to the formation of intrachrosomome bridges as well as to the occurrence of chromosome fragments [16]. It is also possible that the bridges result from chromosome adhesions which, in this case, are multiple and persist until telophase [17]. Our data agree with this statement since we noted sequences of cell divisions showing adhesion-mediated bridges which later suffered chromosome rearrangements in which fragments were duplicated to the same chromosome and segregated to the same cell pole, thus resulting in a cell

with unbalanced chromosomes (Figure 4). Many chromosome fragments were also found scattered within the cell. These fragments probably resulted from simple or double breaks as well as from anaphase bridges that later suffered breaks.

The chromosome rearrangements observed in the present study might be the events responsible for the cumulative mutagenic effect, as described above for the effect of chromium with respect to alterations in the mitotic index. Thus, the clastogenic effect of chromium might lead to cumulative changes in the cell division of cell generations following those of cells exposed to the metal.

Although the Sapucaizinho river in the Municipality of Patrocínio Paulista/SP is considered to be a good quality river whose water is used for different activities, the permitted concentrations of heavy metal residues in effluents emitted by the leather tanning industries should be revised by the authorities in charge. Assessment of the river water using the *A. cepa* test system clearly demonstrated the mutagenic risk potential at some collection sites, especially during the dry period when the frequency of clastogenic effects of these water samples was higher. Since chromium is a heavy metal found in tannery effluents, we believe that the alterations observed in the present study resulted from the action of this metal on *A. cepa* cells. All effects were observed in water samples with a chromium concentration below that permitted by the legislation of the State of São Paulo (1995) and the

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World Health Organization (0.05 mg/L) (Tables 3 and 4), indicating that this parameter should be revised in terms of its potential to induce genotoxicity.

Table 1 - Cell changes observed in *Allium cepa* root tip systems submitted to the action of waters contaminated with chromium residues derived from leather tanning ndustries, collected from the Sapucaizinho River in 2001.

period	Treatment	A	B	C	D	E	F	G	H	I
rainy										
	Conrinuous	-								
	CN	-	-	-	-	-	-	-	9.75 a	0.0 a
	CP	0.3	1.2	0.7	0.2	0.4	2.8	-	4.97 b	10.95 b
	PA	1	0.9	0.9	-	-	-	0.8	8.95 a	1.75 a
	PB-A	1.1	0.9	1	-	-	0.3	2.2	8.49 a	0.82 a
	PB-T	1	1	1	-	-	0.3	1.3	10.76 a	1.29 a
	PB-L	1.5	0.9	0.5	-	-	-	2.1	9.50 a	1.25 a
	PC	1	1.1	0.6	-	0.1	-	1.8	10.50 a	1.45 a
	20 hours									
	CN	-	-	-	-	-	-	-	9.75 a	0.0 a
	CP	0.3	1.2	0.7	0.2	0.6	2.8	-	4.97 b	10.95 b
	PA	0.6	0.4	0.4	-	0.1	0.7	0.2	8.62 a	2.72 a
	PB-A	2.1	0.25	0.1	-	-	0.1	0.3	7.07 a	2.20 a
	PB-T	0.7	0.2	0.1	0.1	0.1	-	1	6.33 a	1.96 a
	PB-L	1.7	0.2	0.2	0.1	0.1	0.2	0.8	8.89 a	1.35 a
	PC	1	0.5	0.4	-	0.2	-	1.1	9.43 a	2.22 a
	72 hours									
	CN	-	-	-	-	-	-	-	9.75 a	0.0 a
	CP	0.3	1.2	0.7	0.2	0.6	2.8	-	4.97 b	10.95 b
	PA	0.3	0.3	0.2	-	-	-	0.5	8.91 a	0.55 a
	PB-A	0.4	0.4	0.2	-	-	0.1	0.7	8.34 a	0.71 a
	PB-T	1	0.2	0.1	-	-	-	0.3	4.93 a	0.50 a
	PB-L	0.9	0.3	0.2	0.4	-	0.1	1	7.18 a	1.60 a
	PC	0.2	0.1	-	-	-	-	0.1	5.37 a	0.47 a
dry										
	Continuous									
	CN	0.6	0.1	-	-	0.7	-	1.2	8.91 a	0.52 c
	CP	1.2	1.8	2.2	0.1	3	7.9	-	5.72 b	18.07 a
	PA	0.7	0.2	0.3		0.4	-	0.9	8.62 a	1.61 bc
	PB-A	0.7	0.4	0.3	0.4	0.3	0.1	1.5	8.92 a	3.16 b
	PB-T	0.6	0.2	0.4	1.1	0	0.2	2.3	6.86 ab	6.06 b
	PB-L	3.1	0.7	0.6	1.8	0.3	1	4.8	8.82 a	3.85 b
	PC	0.9	0.5	0.31	0.5	0.7	-	2.3	7.62 ab	3.03 b
	20 hours									
	CN	0.6	0.1	-	-	0.7	-	1.2	8.91 a	0.52 c
	CP	1.2	1.8	2.2	0.1	3	7.9	-	5.72 cd	18.07 a
	PA	1.5	0.2	0.3	0.7	0.6	0.1	1.9	8.11 ab	2.06 bc
	PB-A	1.4	0.2	0.1	0.8	0.2	-	1.4	8.59 ab	1.52 bc
	PB-T	7.6	0.1	4.1	-	-	1.2	5.4	2.95 d	11.78 bc
	PB-L	1.6	0.5	0.7	1.7	1.3	-	4.2	8.39 ab	4.84 b
	PC	0.8	0.3	0.4	1.7	0.7	-	2.1	5.9 d	2.99 b
	72 hours									
	CN	0.6	0.1	-	-	0.7	-	1.2	8.91 a	0.52 a
	CP	1.2	1.8	2.2	0.1	3	7.9	-	5.72 b	18.07 b
	PA	-	-	-	-	-	-		5.68 b	0.0 a
	PB-A	1.6	0.2	0.1	-	0.2	-	0.5	6.69 ab	0.70 a
	PB-T	2.3	0.4	0.6	-	-	0.1	1.1	5.32 b	1.93 a
	PB-L	1.2	0.2	0.32	0.1	0.4	-	1	6.48 ab	1.56 a
	PC	1.5	0.1	0.3	0.7	0.6	0.1	1.8	5.97 b	2.58 a

A- micronucleus; **B-** anaphase with a bridge; **C-** multipolar anaphase; **D-** delayed anaphase; **E-** sum of chromosome breaks and losses; **F-** sum of C-metaphase and adherence; **G-** binucleate cell; **H-** frequency of the mitotic index; **I-** frequency of aberrant cells in relation to dividing cells. Means followed by the same letters did not d iffer at the 5% level of siginificance (mean rank Kruskal -Wallis test).

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Table 2 - Cell changes observed in *Allium cepa* root tip systems submitted to the action of waters contaminated with chromium residues derived from leather tanning industries, collected from the Sapucaizinho River in 2002.

period	Treatment	A	B	C	D	E	F	G	H	I
rainy										
	Continuous									
	CN	2	-	-	-	0.9	1.0	-	8.29 a	0.18 d
	CP	1.8	1.6	2.0	-	2.6	5.4	-	1.43 b	15.81 a
	PA	0.3	0.4	0.2	0.2	0.5	1.2	0.2	11.73 a	3.66 c
	PB-A	0.6	0.4	0.5	-	1.2	1.6	0.4	8.05 a	5.30 bc
	PB-T	0.5	0.7	0.2	0.1	1.7	3.2	0.1	7.02 a	9.33 ab
	PB-L	0.4	0.6	0.1	0.3	0.7	1.8	0.1	8.49 a	4.68 bc
	PC	0.1	-	-	-	0.6	1.3	-	6.66 a	2.89 c
	20 hours									
	CN	2	-	-	-	0.9	0.1	-	8.29 a	0.18 c
	CP	1.8	1.6	2.0	-	2.6	5.4	-	1.43 b	15.81 a
	PA	0.5	0.4	0.6	0.1	0.4	1.5	-	6.56 a	4.85 b
	PB-A	0.4	2.5	0.9	-	0.4	1.8	0.1	7.65 a	5.88 b
	PB-T	0.8	-	-	-	-	0.3	0.9	2.64 a	2.57 bc
	PB-L	0.2	0.8	0.3	0.1	0.7	2.0	0.1	7.31 a	5.83 b
	PC	0.2	0.4	0.2	-	0.1	1.0	0.3	7.5 a	3.00 bc
	72 hours									
	CN	2	-	-	-	0.9	0.1	-	8.29 a	0.18 d
	CP	1.8	1.6	2.0	-	2.6	5.4	-	1.43 b	15.81 a
	PA	0.4	0.3	-	-	0.9	2.6	0.2	5.81 a	6.49 bc
	PB-A	0.4	0.3	0.1	0.1	0.5	1.1	0.6	6.38 a	2.65 cd
	PB-T	2.0	0.6	0.7	0.1	0.6	2.8	0.3	5.22 a	9.64 ab
	PB-L	0.7	1.0	0.6	0.1	0.9	1.9	0.1	6.11 a	8.51 ab
	PC	0.6	0.2	0.2	-	0.1	1.2	-	5.82 a	3.67 bc
	Recovery									
	CN	2	-	-	-	0.9	0.1	-	8.29 a	0.18 c
	CP	1.8	1.6	2.0	-	2.6	5.4	-	1.43 a	15.81 a
	PA	0.5	-	-	-	0.7	2.1	-	4.7 a	6.84 b
	PB-A	0.8	0.1	0.3	0.2	1.1	0.6	-	5.03 a	4.92 b
	PB-T	2.0	0.4	0.2	0.2	0.9	1.0	-	4.97 a	5.08 b
	PB-L	-	0.1	-	-	0.2	0.2	-	2.21 a	4.02 bc
	PC	0.9	0.5	0.4	0.1	0.3	1.9	0.3	4.33 a	8.23 ab
dry										
	Continuous									
	CN	2.2	1.6	-	-	-	-	-	11.36 a	2.04 c
	CP	4.4	2.2	0.8	0.9	1	5.1	0.1	6.81 b	14.42 a
	PA	2.0	1.0	0.3	0.4	0.7	2.4	0.1	8.24 a	7.37 bc
	PB-A	1.1	0.3	0.2	0.1	1.5	1.5	0.1	7.64 a	5.17 b
	PB-T	1.1	0.6	1.0	0.3	2.1	1.4	0.1	7.74 ab	5.65 b
	PB-L	1.3	1.5	1.1	0.2	1.2	0.8	0.3	7.54 a	5.62 b
	PC	1.4	0.4	0.2	0.1	1.0	1.0	0.3	7.47 ab	3.94 b
	20 hours									
	CN	2.2	1.6	-	-	-	-	-	11.36 a	2.04 c
	CP	4.4	2.2	0.8	0.9	1.0	5.1	0.1	6.81 cd	14.42 a
	PA	1.2	0.5	0.2	-	1.6	2.1	0.2	5.56 ab	5.85 bc
	PB-A	0.2	1.1	0.8	-	2.8	3.8	-	7.17 ab	6.32 bc
	PB-T	0.6	0.4	0.4	-	0.1	2.3	0.4	4.21 d	4.32 bc
	PB-L	0.4	1.9	0.9	-	0.8	2.1	-	6.45 ab	5.22 b
	PC	1.0	1.1	0.8	-	1.5	2.7	0.2	7.58 bc	5.61 b
	72 hours									
	CN	2.2	1.6	-	-	-	-	-	11.36 a	2.04 b
	CP	4.4	2.2	0.8	0.9	1.0	5.1	0.1	6.81 b	14.42 a
	PA	1.1	0.5	1.2	0.2	0.3	3.4	0.1	7.17 b	5.52 b
	PB-A	1.2	1.0	0.2	-	1.4	3.7	0.1	7.21 b	5.65 b
	PB-T	3.6	1.2	0.6	-	1.2	1.5	0.3	4.87 b	9.23 b
	PB-L	1.3	2.3	0.9	-	3.1	3.3	-	7.31 ab	8.42 b
	PC	1.2	0.3	0.6	0.4	2.1	6.1	-	6.39 b	9.62 b
	Recovery									
	CN	2.2	1.6	-	-	-	-	-	11.36 a	2.04 c
	CP	4.4	2.2	0.8	0.9	1.0	5.1	0.1	6.81 a	14.42 a
	PA	1.8	0.5	0.3	0.1	1.9	1.6	0.6	4.53 a	4.82 c
	PB-A	0.9	-	0.2	0.2	1.4	0.3	-	6.26 a	3.57 c
	PB-T	2.1	1.1	0.6	1.4	2.1	4.3	0.3	6.43 a	11.69 ab
	PB-L	1.5	1.6	1.4	0.4	4.4	2.9	0.2	6.42 a	11.72 ab
	PC	1.8	1.0	0.7	0.2	2.9	2.9	-	3.92 a	8.38 bc

A- micronucleus; **B-** anaphase with a bridge; **C-** multipolar anaphase; **D-** delayed anaphase; **E-** sum of chromosome breaks and losses; **F-** sum of C-metaphase and adherence; **G-** binucleate cell; **H-** frequency of the mitotic index; **I-** frequency of aberrant cells in relation to dividing cells. Means followed by the same letters did not d iffer at the 5% level of siginificance (mean rank Kruskal-Wallis test).

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Table 3 - Chemical analysis of the water (results are reported as ppm) collected at the different sites along the Sapucaizinho river in 2001.

		Chemical elements										
		Pb	Fe	Cd	Cr	P	Al	Zn	Cu	Ba	Co	Ni
Summer												
	PA	<0.025	1.2	<0.005	<0.01	0.1	0.06	0.02	0.01	0.03	<0.01	<0.01
	PB-A	<0.025	2.0	<0.005	<0.01	<0.1	0.10	0.03	<0.01	0.03	<0.01	<0.01
	PB-T	<0.025	0.2	<0.005	0.37	0.5	0.7	0.06	0.01	0.005	<0.01	0.01
	PB-L	<0.025	1.8	<0.005	<0.01	0.1	0.17	0.05	0.01	0.03	<0.01	<0.01
	PC	<0.025	0.7	<0.005	<0.01	2.5	0.30	0.05	<0.01	0.05	<0.01	<0.01
Autumn												
	PA	<0.025	1.3	<0.005	<0.01	<0.1	<0.05	<0.01	<0.01	0.02	<0.01	<0.01
	PB-A	<0.025	1.0	<0.005	<0.01	<0.1	0.06	0.01	<0.01	0.02	<0.01	<0.01
	PB-T	<0.025	0.2	<0.005	0.04	0.15	0.35	0.01	<0.01	0.01	<0.01	<0.01
	PB-L	<0.025	1.1	<0.005	<0.01	<0.1	0.10	0.09	<0.01	0.02	<0.01	<0.01
	PC	<0.025	0.8	<0.005	<0.01	<0.1	<0.05	<0.01	<0.01	0.02	<0.01	<0.01
Winter												
	PA	<0.025	0.27	<0.005	<0.01	<0.1	<0.05	<0.01	<0.01	0.02	<0.01	<0.01
	PB-A	<0.025	0.07	<0.005	<0.01	<0.1	<0.05	<0.01	<0.01	0.30	<0.01	<0.01
	PB-T	<0.025	1.4	<0.005	0.21	0.19	0.4	0.03	0.03	0.20	<0.01	0.01
	PB-L	<0.025	0.08	<0.005	<0.01	<0.1	<0.05	<0.01	<0.01	0.02	<0.01	<0.01
	PC	<0.025	0.10	<0.005	<0.01	<0.1	<0.05	<0.01	<0.01	0.02	<0.01	<0.01
Spring												
	PA	<0.025	0.61	<0.005	<0.01	<0.1	<0.05	<0.01	<0.01	0.02	<0.01	<0.01
	PB-A	<0.025	0.32	<0.005	<0.01	<0.1	<0.05	<0.01	<0.01	0.02	<0.01	<0.01
	PB-T	<0.025	0.09	<0.005	0.45	0.10	0.24	0.02	<0.01	0.01	<0.01	0.01
	PB-L	<0.025	0.42	<0.005	0.01	<0.1	<0.05	<0.01	<0.01	0.02	<0.01	<0.01
	PC	<0.025	0.37	<0.005	<0.01	<0.1	<0.05	<0.01	<0.01	0.02	<0.01	<0.01

PA- upstream of the river; **PB-A**- 3m before the disposal site; **PB-T**- effluent water before disposal into the river; **PB-L**- disposal site; **PC**- downstream of the river

Table 4 - Chemical analysis of the water (results are reported as ppm) collected at the different sites along the Sapucaizinho river in 2002.

		Chemical elements										
		Pb	Fe	Cd	Cr	P	Al	Zn	Cu	Ba	Co	Ni
Summer												
	PA	<0.025	1.1	<0.005	<0.01	<0.1	0.12	<0.01	<0.01	0.02	<0.01	<0.01
	PB-A	<0.025	1.1	<0.005	<0.01	<0.1	0.09	<0.01	<0.01	0.03	<0.01	<0.01
	PB-T	<0.025	0.13	<0.005	0.2	0.51	5.6	0.03	<0.01	0.05	<0.01	0.01
	PB-L	<0.025	1.2	<0.005	0.01	<0.1	0.2	<0.01	<0.01	0.03	<0.01	<0.01
	PC	<0.025	1.1	<0.005	<0.01	<0.1	0.1	<0.01	<0.01	0.02	<0.01	<0.01
Autumn												
	PA	<0.012	0.96	<0.003	<0.005	<0.1	0.05	<0.005	<0.005	0.02	<0.005	<0.005
	PB-A	<0.012	0.92	<0.003	<0.005	<0.1	0.06	<0.005	<0.005	0.03	<0.005	<0.005
	PB-T	<0.012	0.12	<0.003	0.2	0.26	0.35	0.01	<0.005	0.01	0.01	0.005
	PB-L	<0.012	0.81	<0.003	0.005	<0.1	0.1	<0.005	<0.005	0.03	<0.005	<0.005
	PC	<0.012	0.85	<0.003	<0.005	<0.1	0.07	<0.005	<0.005	0.02	<0.005	<0.005
Winter												
	PA	<0.02	0.57	<0.003	<0.005	<0.1	0.1	<0.005	<0.005	0.02	<0.005	<0.005
	PB-A	<0.02	0.35	<0.003	<0.005	<0.1	0.05	<0.005	<0.005	0.02	<0.005	<0.005
	PB-T	<0.02	0.29	<0.003	<0.005	0.43	0.03	0.01	<0.005	0.02	0.005	0.005
	PB-L	<0.02	0.13	<0.003	0.17	0.48	0.35	0.01	<0.005	0.01	<0.005	<0.005
	PC	<0.02	0.43	<0.003	<0.005	<0.1	0.08	<0.005	<0.005	0.02	<0.005	<0.005
Spring												
	PA	<0.02	2.6	<0.003	<0.005	<0.1	4.17	0.01	0.02	0.02	<0.005	<0.005
	PB-A	<0.02	2.77	<0.003	<0.005	<0.1	4.33	0.01	0.01	0.01	<0.005	<0.005
	PB-T	<0.02	0.16	<0.003	0.39	0.49	1.04	0.03	0.01	0.02	0.005	0.01
	PB-L	<0.02	3.15	<0.003	<0.005	0.11	5.27	<0.005	0.02	0.02	<0.005	<0.005
	PC	<0.02	3.24	<0.003	<0.005	0.11	5.23	<0.005	0.01	0.01	<0.005	<0.005

PA- upstream of the river; **PB-A**- 3m before the disposal site; **PB-T**- effluent water before disposal into the river; **PB-L**- disposal site; **PC**- downstream of the river

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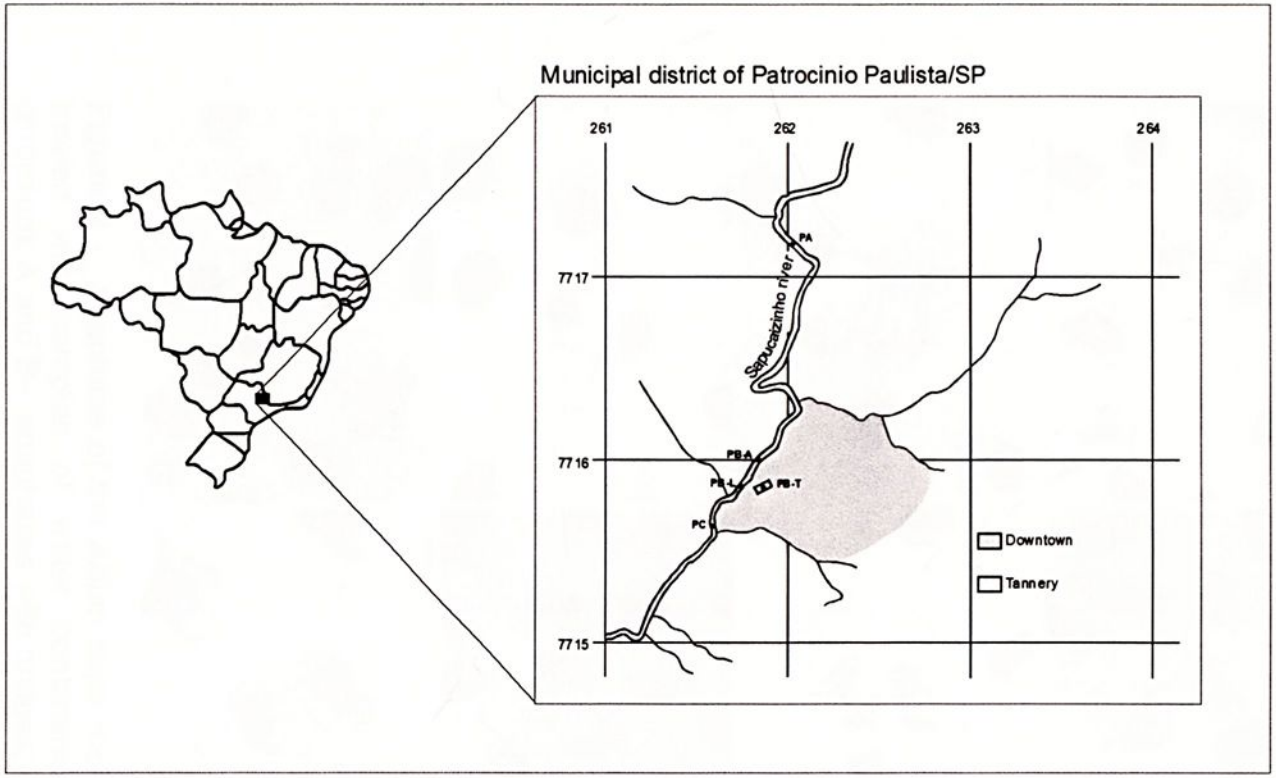

Figure 1 - Geographical localization of the collected site along the Sapucaizinho river.

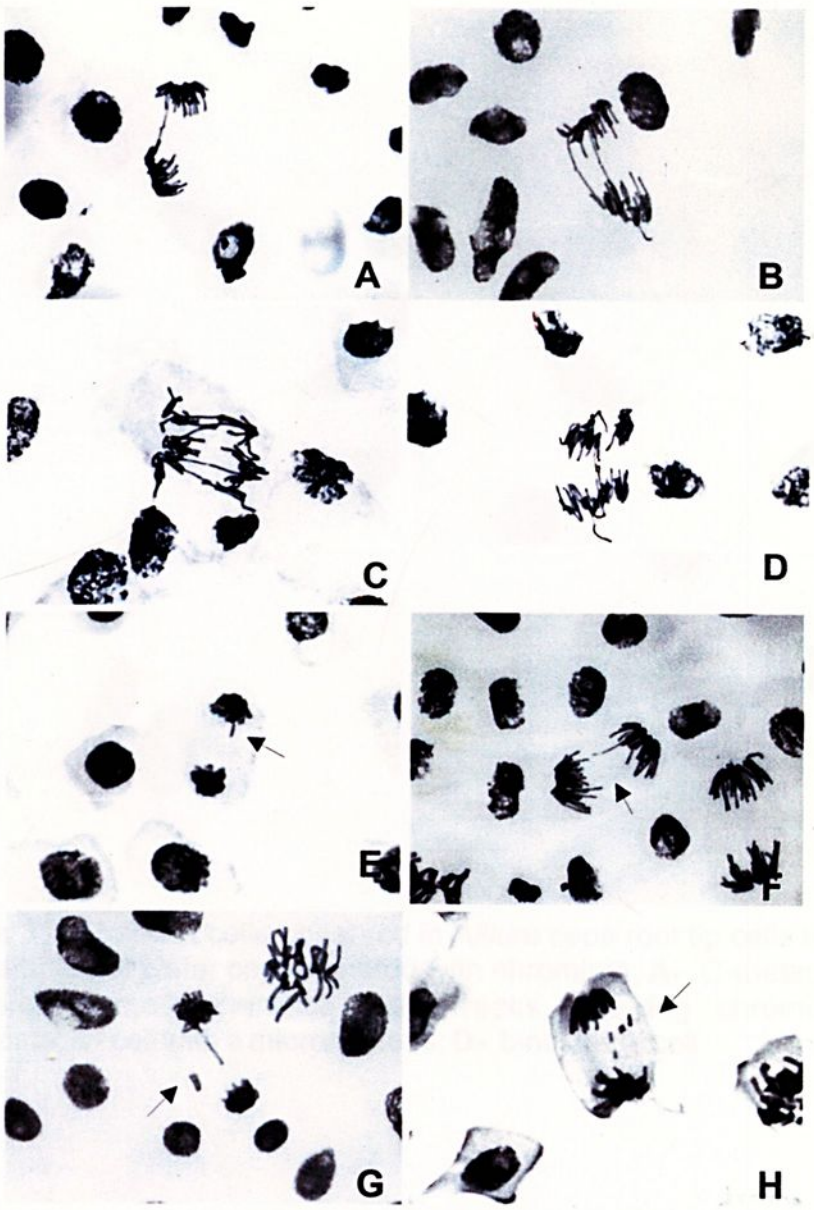

Figure 2 - Anaphase of the *Allium cepa* root tip cells treated with samples of water contaminated with chromium: **A** and **B-** anaphases with bridges; **C** and **D-** multipolar anaphases; **E-** cell with laggard chromosomes; **F**, **G** and **H-** cells with breaks demonstrating chromosome fragments.

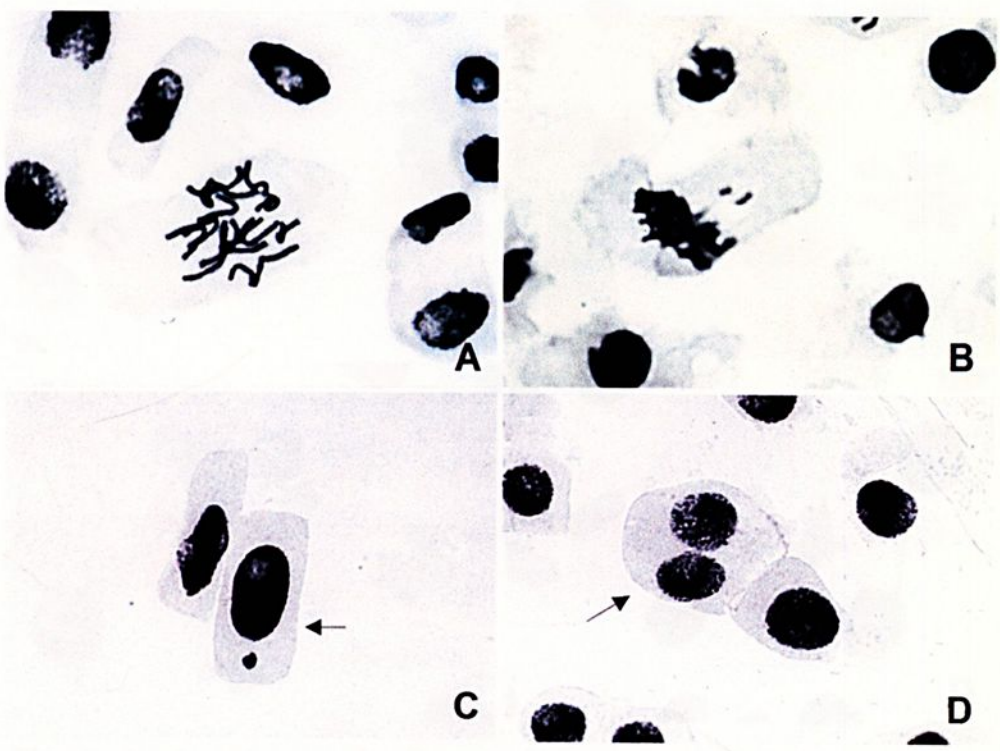

Figure 3 - Aberrant cells observed in *Allium cepa* root tip cells treated with samples of water contaminated with chromium. **A-** C-metaphase; **B-** chromosome adherence with breaks, showing chromosome fragments; **C-** cell with a micronucleus; **D-** binucleate cell.

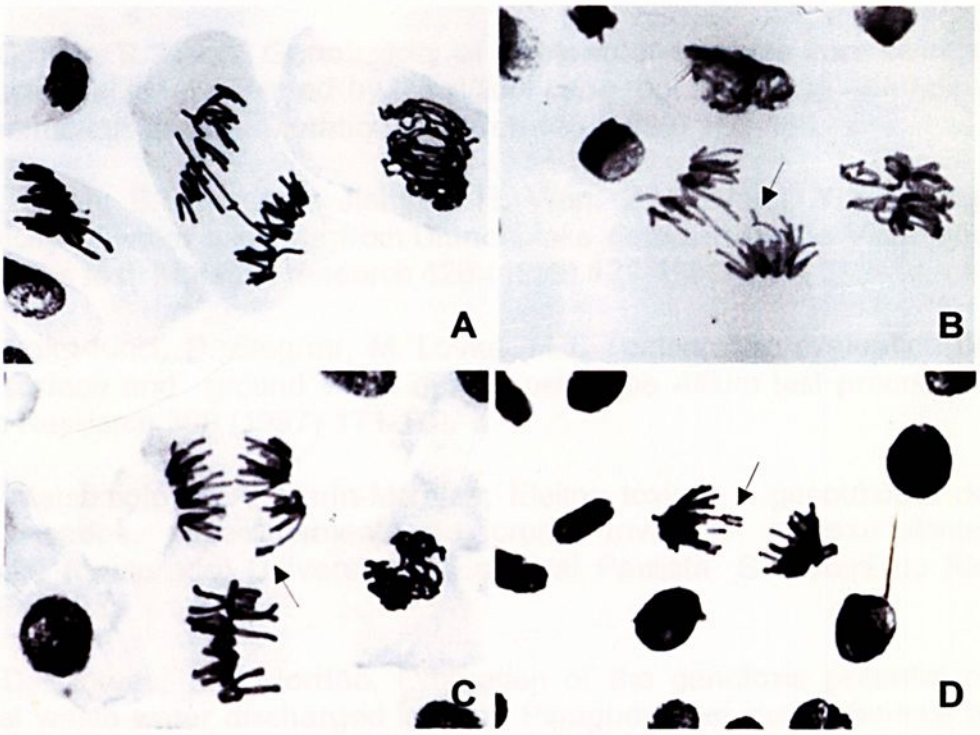

Figure 4 - Anaphases of cells submitted to germination in samples of water contaminated with chromium residues. **A-** Anaphase with bridges showing telomere adherence; **B**, **C** and **D-** anaphases with chromosomes duplicated in the telomeric portions due to chromosome adherence taking place in the previous phase of the cell cycle.

References

[1] R.M. Harden, The learning environment and the curriculum. Medical teacher 23 (2001) 335-336

[2] I.S. Grover, S. Kaur, Genotoxicity of wastewater samples from sewage and industrial effluent detected by the *Allium cepa* root anaphase aberration and micronucleus assays. Mutation research 426 (1999) 183-188.

[3] C.Q. Duan, B. HU, X.H. Jiang, C.H. Wen, Z.H. Wang, Y.X. Wang, Genotoxicity of water samples from Dianchi lake detected by the Vicia faba micronucleus test. Mutation research 426 (1999) 121-125.

[4] V. Smaka-Kincl, P. Stegnar, M. Lovka, M.J. Toman, The evaluation de waster, surface and ground water quality using the *Allium* test procedure. Mutation Research 368 (1997) 171-179.

[5] S.T. Matsumoto, M.A. Marin-Morales, Efeitos tóxicos e genotóxicos de metais pesados, especificamente do cromo trivalente e hexavalente. Monografia (doutorado) Universidade Estadual Paulista. São José do Rio Preto-SP (2003).

[6] D.S.D. Moraes, B.Q. Jordão, Evaluation of the genotoxic potential of municipal waste water discharged into the Paraguay river during periods of flood and drought. Environmental toxicology 16 (2001) 113-116.

[7] T. O'Brien, J. Xu, S.R. Patierno, Effects of glutathione on chromium-induced DNA crossinking and DNA polymerase arrest. Molecular and cellular biochemistry 222 (2001) 173-182.

[8] R. Codd, C.T. Dillon, A Levina, Studies on the genotoxicity of chromium: from the test tube to the cell. Coordination Chemistry Reviews 216 (2001) 537-582.

[9] A. Leonard, R.R. Lauwerys Carcinogenicity and mutagenicity of chromium. Mutation research 76 (1980) 227-239.

[10] E.T. Snow, Metal carcinogenesis: mechanistic implications. Pharmacology and therapeutics 53 (1992) 31-65.

[11] K.D. Sugden, R.B. Burris, S.J. Rogers, An Oxygen Dependence In Chromium Mutagenesis. Mutation research 244 (1990) 239-244.

F. 7588/04

[12] A.N. Sahi, S.K. Singh, P.K. Sem, R.N. Singh, Gytogenetic response of hexavalent shromium-induced somatic cell abnormalities in *Allium cepa* Cytobios 96 (1998) 71-79

[13] D.H. Liu, W.S. Jiang, M.X. Li, Effects of trivalent and hexavalent chromium on root-growth and cell-division of allium-cepa. Hereditas 117 (1982) 23-29.

[14] S. Gomez-arroyo, R. Vallalobos-Spietrini, chromosomal alterations induced by some chromium salts. Cytologia 48 (1983) 185-203.

[15] M.A.H. Franson, Standard Methods for the Examination of Water and Wastewater. 19th Ed. Washington (1995).

[16] G. Fiskesjö, The *Allium cepa* in wastewater monitoring. Environmental toxicology and water quality: An International Jounal 8 (1993) 291-298

[17] F.R.B. Giacomelli, Avaliação do comportamento meiótico em variedades de Aveia (*Avena sativa*) recomendadas para a região Sul. Dissertação (mestrado). Universidade Estadual de Maringá. Maringá-PR. (1999).

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5. – ARTIGO 03

EVALUATION OF THE CLASTOGENIC EFFECT OF THE WATERS OF A STREAM THAT RECEIVES TANNERY EFFLUENTS, BY ANALYSIS OF *Allium cepa*

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Abstract

Chromium compounds induce a variety of damages to the DNA molecule, including single and double chain breaks which generate chromosomal aberrations, micronucleus formation, sister chromatid exchanges, formation of DNA adducts, and replication and transcription errors. Water samples were collected on a seasonal basis for the Bagres Stream, Municipality of Franca, State of São Paulo, Brazil, along which several leather tanneries are installed. The cytotoxic and genotoxic potential of these waters was assessed by analysis of the cell cycle of *Allium cepa* root tip cells. The most frequent chromosomal abnormalities observed in these analyses were: c-metaphases, chromosome adherence, chromosome breaks and losses, anaphase with a bridge and multipolar, cells with micronuclei and binucleate cells. The frequencies of the cytotoxic and genotoxic effects on *Allium cepa* root tip cells were higher at the collection sites where the total chromium concentration was also higher.

Key words: *Allium cepa*, genotoxicity, Chromium

cm 1 2 3 4 5 6 7 8 unesp 12 13 14 15 16 17 18

Introduction

The presence of genotoxic agents in rivers due to the contamination provoked by human actions such as industrial, agricultural and domestic activities can be estimated by means of analyses of changes in the cell cycle.

Chromium compounds possess confirmed toxic, genotoxic, mutagenic and carcinogenic effects on man and animals (Von Burg and Liu, 1993; Stohs and Bagchi, 1995; Mount and Hockett, 2000). Active chromium compounds (trivalent-Cr III and hexavalent- Cr VI) differ from one another in their ability to cross biological membranes. Cr III has an octahedral geometric structure that makes biological membranes more impermeable to it than Cr VI, whose structure is tetrahedral (Leonard and Lauwerys, 1980; Beyersmann *et al.*, 1994; Stohs and Bagchi, 1995; Singh *et al.*, 1998; Matsumoto, 2003). However, if chromium III has access to the intracellular medium through processes such as pinocytosis and endocytosis or by the reduction of Cr VI, it acts directly on DNA, damaging it in a more efficient manner than done by chromium VI (Matsumoto, 2003).

Several *in vivo* and *in vitro* studies have shown that chromium compounds induce a variety of damages to the DNA molecule, including single and double chain breaks that generate chromosomal aberrations, micronucleus formation, sister chromatid exchanges, formation of DNA

adducts, and replication and transcription errors (Zhitkovich *et al.*, 1996; O'Brien *et al.*, 2001; Matsumoto, 2003; Matsumoto *et al.*, *in press*).

In mutagenesis, the choice of a test organism should follow some criteria that will permit the safe evaluation of the potential of a given agent to induce changes in genetic material. Some of these changes can be assessed by the structural and numerical modifications they promote in the chromosomes, resulting in chromosome aberrations.

An efficient test organism for the assessment of chromosomal aberrations should have chromosomes easy to analyze in terms of size, morphology and number. The higher plants *Allium cepa*, *Tradescantia paludosa* and *Vicia faba* have relatively large monocentric chromosomes in reduced numbers, and are indicated as test organisms for the study of environmental mutagenesis (Rank and Nielsen, 1998; Grover and Kaur, 1999; Kong and Ma, 1999; Haroun and Shehri, 2001; Moraes and Jordao, 2001; Patra and Sharma, 2002; Matsumoto *et al.*, *in press*).

Grover and Kaur (1999) and Patra and Sharma (2002) demonstrated the clastogenic and aneugenic effect of atmospheric, water and soil pollutants by means of the micronucleus assay in *A. cepa* and *V. faba* root tip cells.

The *A. cepa* test system was used by Grover and Kaur (1999), Sudhakar *et al.* (2001) and Matsumoto *et al.* (*in press*) to investigate the mutagenic potential of industrial effluents. According to these authors, the chromosomal changes most frequently observed were c-metaphases,

chromosomal adherence and breaks, bridges, laggards, binucleate cells and cells with micronuclei.

Using *Allium cepa* test systems, Sahi *et al.* (1998) assessed the effects of chromium contamination on the waters of an Indian river and showed that at the sites where chromium concentrations were high there was a reduction in mitotic index and an increase in the rate of mitotic abnormalities, thus confirming the cytotoxic and genotoxic effect of the metal.

In the municipality of Franca, State of São Paulo, Brazil, there are several leather tanneries. During the tanning procedure, Cr III is used as raw material. The objective of the present study was to assess the cytotoxic and genotoxic potential of the waters of a stream in the Franca region (Bagres Stream) which receives tannery effluents by analyzing the cell cycle of *A. cepa* root tip cells.

Materials and Methods

Seasonal water collections were performed in the years 2001 and 2002 at three distinct sites along the Bagres Stream, municipality of Franca, SP, Brazil. The sites selected for water collection were standardized as follows: 200 m upstream of the site of discharge of the tannery effluent (FA), exactly at the site of discharge (FB), and 500 downstream from the site of discharge (FC) (Figure 1).

The analyses were based on the Standard Methods for the Examination of Water and Wastewater (Franson, 1995).

For cation determination, the samples were first acidified with HNO_3, pH 1, and the cations were analyzed sequentially by inductively coupled plasma atomic emission spectrometry (ICP-AES) with ultrasonic nebulization. The following elements were determined: calcium, magnesium, strontium, silicon, iron, manganese, aluminum, zinc, chromium, cobalt, nickel, lead, cadmium, phosphorus, copper, and barium.

The standard solutions used to construct the calibration curves for the elements analyzed in 0.1% HNO_3 medium were obtained by appropriate dilutions of the standard solutions of 1000 ppm titrisol (Merck).

A. cepa seeds were submitted to germination in the waters collected at the cited sites. The negative control consisted of seeds submitted to germination in Milli-Q water and the positive control consisted of an aqueous solution of trivalent chromium (0.089 mg/L). The tests were carried out using seeds of a single variety of *A. cepa* to prevent the possible occurrence of different responses to the various steps of the procedure.

Two types of treatments were applied: A) continuous treatment consisting of seed soaking and germination in the waters obtained at the collection sites, and B) discontinuous treatment consisting of seeds first soaked in Milli-Q water until they reached 2 cm in length and later transferred to a Gerbox containing the waters from the collection sites, where they were

left for 20 hours (acute treatment). After this time, some roots were collected at random and the remaining roots were left under the same conditions until they completed 72 hours of treatment (chronic treatment), after which they were collected. Only for the collections performed in 2002, after chronic treatment some roots were transferred to plates containing Milli-Q water and left there for 48 hours (recovery treatment), after which they were collected.

All collected root tips were fixed in 3:1 Carnoy (3 parts ethanol and 1 part acetic acid) for 24 hours.

For cytological analysis, all slides were prepared by the common method of gentle squashing, with the root tips being submitted to acid hydrolysis with 1 N HCl at 60ºC for 8 minutes, followed by washing with distilled water. The material was submitted to Schiff staining for 2 hours in the dark. All cells with alterations were counted and the most representative ones for each abnormality were photographed.

For analysis of cell division rates, slides with root tip cells from both treatments were prepared by the methodology described above. The percentages of cells in each phase of cell division were determined.

For the assessment of genotoxic potential, dividing cells with irregular anaphases (disorganized, multipolar or laggard anaphases), cells with chromosome adherence, cells with micronuclei, and binucleate and/or multinucleate cells were analyzed.

Data were analyzed statistically by the Kruskal-Wallis test.

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Results and Discussion

All water samples collected were assayed for the assessment of the cytotoxic potential of the chemical substances present at different sites along the Bagre Stream. The potential of these waters to induce alterations of the mitotic index was estimated by the *Allium cepa* test. According to Smaka-Kincl *et al.* (1997), the frequency of the mitotic index may be reduced or increased according to the degree of cytotoxicity and genotoxicity of the samples under study.

The mitotic indices of *A. cepa* root tip cells treated with water samples collected in 2001 were lower than those observed in the negative control both during the dry and the rainy periods, except for the FA site (continuous treatment, rainy season, 10.09) (Table 1).

The mitotic indices obtained after treatment with water samples from the FB site (site of effluent discharge) and FC site (downstream from the discharge site) collected during the dry season in 2002 did not differ from one another or from the positive control for any of the treatments performed (Table 2). These data demonstrate the cytotoxic effect of the water obtained at these sites, probably due to the chromium residues present in the water during the dry season. These sites correspond to the places receiving the impact of discharge of the tannery effluents, with consequent contamination with chromium.

For the rainy season of 2002, analysis of the mitotic indices revealed that none of the sites tested was contaminated with amounts of residues capable of promoting changes in the mitotic index of the test organisms, since the values obtained were lower than those for the negative control. No significant differences were observed between the negative control, the sites of continuous treatment, the FA and FC sites of acute (20 hours) discontinuous treatment and the FA site of discontinuous chronic treatment (Table 2). These data demonstrate that there was no evidence of a cytotoxic effect of the waters collected during the rainy season at the FA site (acute and chronic discontinuous treatment). This result may be explained by the marked dilution suffered by the effluents during the rainy season, since water flow increases considerably in the river during this period, and also by the location of the FA site itself (a site along the river that does not suffer any impact from the toxic residues released by the tannery effluents). Thus, these data confirm that the mitotic index represents a good parameter for the analysis of the cytotoxic effect of contaminants present in aquatic environments.

For the recovery treatment, performed with samples collected during the dry period and with the positive control, no significant difference was observed between assays (Table 2). These data indicate that, with the return to normal environmental conditions, a potential recovery of the mitotic index altered by chromium can occur. Nacci et al. (1992) also reported a reduction

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at damage in organisms transferred from sites with sediments contaminated with heavy metals to control sites free from these pollutants. On the basis of the similarity of the response observed in the assays of waters from sites contaminated with chromium and of the positive control (an aqueous solution of chromium), it seems clear that chromium is the possible contaminant present along the stream, capable of inducing cytotoxic effects on *Allium cepa* root tip cells.

No assays were performed to assess the potential for recovery of mitotic indices at any of the sites studied in the year 2001.

To estimate the genotoxic effect of the water samples collected, the frequencies of aberrant cells among dividing cells were assessed. For all the collections at all sites evaluated (dry and rainy seasons of 2001 and 2002), the rates of aberrant cells were higher than those recorded for the negative control (Tables 1 and 2).

For the dry period of 2001, genotoxic and clastogenic effects were recorded for the water samples from the FB site – continuous treatment, and the FA and FB sites – discontinuous treatment (20 hours).

During the rainy season of 2001, the genotoxic effect was observed only for the FB site - chronic discontinuous treatment for 72 hours. This site represents the place where the tannery effluent is received, where the concentration of total chromium residue was higher (0.38, 0.11 and 0.07) than the maximum concentration permitted by the 1995 Legislature of the State of

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São Paulo (0.05 mg/L) (Table 3). These results support the suggestion that chromium is the contaminant present in the water samples of the Bagres Stream and is responsible for the clastogenic action on *A. cepa* since only the water from the sites located downstream from the discharge of the tannery effluent demonstrated this effect, except for the FA site (discontinuous treatment for 20 hours).

For the water samples collected in 2002 both during the dry and rainy seasons, the frequency of aberrant cells was high for the FB and FC sites (Table 2). Since these sites contain chromium residues derived from the emission of the tannery effluent, this may support the suggestion that the genotoxicity observed is due to the pollutants of these effluents, and chromium in particular (Table 4).

The most frequent aberrations observed in the present analyses were c-metaphases, chromosome adherence, anaphase irregularities (dots and multipolarity), chromosome breaks, and cells with micronuclei or binucleate (Tableas 1 and 2, Figure 2, 3 and 4).

Fiskejö (1993) and Sudhakar *et al.* (1998) reported that chromosome breaks may result in the formation of acentric fragments which will give origin to micronuclei over the subsequent cell generations. According to Ma *et al.* (1995), the micronucleus test is a good indicator of the clastogenic effect of toxic substances.

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In the present analyses, some cells presented the loss of whole chromosomes, which persisted up to telophase (Figure 3). These losses would probably result in interphase cells with micronuclei. The present data agree with those obtained by Sudhakar *et al.* (1998), who reported micronucleus induction by involvement of the mitotic spindle, resulting in laggard chromosomes during anaphase and in the loss of the total chromosome.

In the present analyses, we also observed that the frequencies of the cytotoxic, genotoxic and clastogenic effects were higher for the collection sites where total chromium concentration was also higher, confirming the data reported by Sahi *et al.* (1998) and Matsumoto *et al.* (*in press*), who attributed to chromium the potential of inducing chromosome breaks and losses resulting in chromosome aberrations, micronucleus formation, and binucleate cells.

The highest cytotoxic, genotoxic and clastogenic effects were recorded during the dry period (from June to the October). During this period, the water flow is usually low, permitting a grater concentration of chromium. These residues, which have the potential to damage the DNA structure of test organisms (Zhitkovich *et al.*, 1996; O'Brien *et al.*, 2001; Matsumoto, 2003; Matsumoto *et al.*, *in press*), may compromise the quality of the water of the Bagre Stream. These data agree with those obtained by Matsumoto *et al.* (*in press*) who reported a genotoxic effect of the waters of the Bagre Stream

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during the winter season, as determined by the comet assay applied to CHO-K1 cell cultures.

Table 1 - Cell changes observed in *Allium cepa* root tip systems submitted to the action of waters contaminated with chromium residues derived from leather tanning ndustries, collected from the "Corrego dos Bagres" stream in 2001.

period	treatment	A	B	C	D	E	F	G	H	I	J	K
dry												
	continuous											
	CN	0.6	0.1	-	-	-	0.7	-	-	1.2	8.91 a	0.52 c
	CP	1.2	1.8	2.2	0.1	1	2	5.2	2.7	-	5.72 b	18.07 a
	FA	1.1	0.4	0.3	0.4	-	0.6	-	-	1.7	6.34 b	2.61 bc
	FB	0.7	0.4	0.7	0.4	-	0.5	0.1	0.2	2.3	7.19 ab	3.23 b
	FC	0.8	0.3	0.5	0.4	-	0.3	-	0.1	1.6	6.11 b	2.06 bc
	20 hours											
	CN	0.6	0,1	-	-	-	0.7	-	-	1,2	8.91 a	0.52 c
	CP	1.2	1.8	2.2	0.1	1	2	5.2	2.7	-	5.72 b	18.07 a
	FA	1.5	0.6	0.8	0.2	-	0.9	0.1	0.1	2.7	6.93 ab	3.88 b
	FB	1	0.3	1	0.3	-	1.1	-	0.1	2.3	5.56 b	3.85 b
	FC	1.5	0.3	0.4	0.3	-	0.2	-	-	0.3	5.57 b	1.98 bc
	72 hours											
	CN	0.6	0.1	-	-	-	0.7	-	-	1.2	8.91 a	0.52 b
	CP	1.2	1.8	2.2	0,1	1	2	5.2	2.7	-	5.72 b	18.07 a
	FA	0.7	0.1	0.1	0.4	-	-	-	-	0.6	6.53 ab	1.16 ab
	FB	2.1	0.1	0.3	0.4	-	0.1	0.1	-	1	4.97 b	1.98 ab
	FC	1.3	0.1	-	-	-	0.1	-	-	0.2	5.19 b	0.78 ab
rainy												
	continuous											
	CN	-	-	-	-	-	-	-	-	-	9.75 a	0.0 b
	CP	0.3	1.2	0.7	0.2	0.2	0,4	1.1	1.7	-	4.97 b	10.95 a
	FA	0.8	0.4	0.4	-	-	0.2	-	-	1	10.09 a	0.82 ab
	FB	0.4	0.7	0.4	0.2	-	0.1	-	-	0.9	8.43 a	1.47 ab
	FC	1.6	0.5	0.5	-	-	-	-	-	1	8.39 a	1.10 ab
	20 hours											
	CN	-	-	-	-	-	-	-	-	-	9.75 a	0.0 b
	CP	0.3	1.2	0.7	0.2	0.2	0.4	1.1	1.7	-	4.97 c	10.95 a
	FA	0.9	0.3	-	-	-	-	-	-	0.3	7.30 ab	0.87 ab
	FB	2.9	0.1	-	-	-	-	-	0.2	0.3	4.66 b	0.96 ab
	FC	0.3	0.2	0.2	-	0.1	0.1	-	-	0.6	7.65 ab	0.68 ab
	72 hours											
	CN	-	-	-	-	-	-	-	-	-	9.75 a	0.0 c
	CP	0.3	1,2	0.7	0.2	0.2	0.4	1.1	1.7	-	4.97 c	10.95 a
	FA	0.5	0.4	0.3	-	-	-	-	-	0.7	7.53 ab	0.79 ab
	FB	1.6	1	0.1	0.4	0.1	0.4	-	0.1	1.6	4.53 b	11.81 a
	FC	0.3	0.5	0.2	-	-	-	-	-	0.7	6.11 b	2.21 cb

A- micronucleus; **B-** anaphase with bridge; **C-** multipolar anaphase; **D-** delayed anaphase; **E-** chromosome breaks; **F-** losses chromosome; **G-** C-metaphase; **H-** adherence; **I-** binucleate cell; **J-** frequency of the mitotic index; **K-** frequency of aberrant cells in relation to dividing cells. Means followed by the same letters did not d iffer at the 5% level of siginificance (mean rank Kruskal -Wallis test).

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Table 2 - Cell changes observed in *Allium cepa* root tip systems submitted to the action of waters contaminated with chromium residues derived from leather tanning industries, collected from the "Corrego dos Bagres" stream in 2002.

period	Tratamento	A	B	C	D	E	F	G	H	I	J	K
dry												
	Continuous											
	CN	2.2	1.6	-	-	-	-	-	-	-	11.36a	2.04b
	CP	4.4	2.2	0.8	0.9	0.5	0.5	1.2	3.9	0.1	6.81 bc	14.42 a
	FA	0.2	0.8	0.6	-	-	0.2	0.2	0.9	-	8.01 ab	1.88 c
	FB	1.4	0.1	0.2	0.2	-	1	1	2.4	0.6	6.59 c	5.72 ab
	FC						-				3.74 c	3.51 bc
	20 hours											
	CN	2.2	1.6	-	-	-	-	-	-	-	11.36a	2.04b
	CP	4.4	2.2	0.8	0.9	0.5	0.5	1.2	3.9	0.1	6.81 b	14.42 a
	FA	1	0.6	0.8	0.2	1.6	0.9	1	1.8	-	7.41 b	5.49 bc
	FB	0.2	2.2	1.3	0.2	0.6	1	2.3	2.3	-	7.11 b	7.58 b
	FC	0.2	0.7	0.9	-	0.2	0.8	0.9	3.2	-	7.32 b	4.86 bc
	72 hours											
	CN	2.2	1.6	-	-	-	-	-	-	-	11.36a	2.04b
	CP	4.4	2.2	0.8	0.9	0.5	0.5	1.2	3.9	0.1	6.81 b	14.42 a
	FA	2.2	0.9	0.2	-	0.9	1	1.6	1.8	0.2	7.43 ab	6.57 bc
	FB	0.6	-	0.2	-	0.4	0.2	2.8	5.9	0.1	3.64 c	11.93 abc
	FC	2.3	0.5	0.2	-	0.1	0.1	0.5	4.7	-	5.47 bc	7.62 bc
	Recovery											
	CN	2.2	1.6	-	-	-	-	-	-	-	11.36a	2.04b
	CP	4.4	2.2	0.8	0.9	0.5	0.5	1.2	3.9	0.1	6.81 a	14.42 a
	FA	1.0	0.3	1.0	-	-	0.6	0.6	-	0.3	1.77 a	3.35 b
	FB	1.0	1.0	0.4	1.0	1.4	5.2	6.8	2.20	0.2	5.05 a	23.67 a
	FC	1.9	-	-	0.75	1.0	0.82	1.15	1.45	0.4	5.15 a	9.78 a
rainy												
	Continuous											
	CN	2	-	-	-	0.8	0.1	0.1	-	-	8.29 a	0.18 c
	CP	1.8	1.6	2.0	-	1.8	0.8	4.4	1.0	-	1.43 b	15.81 a
	FA	0.3	0.7	0.6	-	1.1	0.5	0.4	0.3	0.1	8.37 a	5.07 b
	FB	-	0.7	0.2	0.1	0.1	0.2	0.6	0.2	-	5.99 a	3.79 a
	FC	0.2	0.8	0.4	0.1	0.1	0.4	1.3	1	1	6.68 a	7.69 ab
	20 hours											
	CN	2	-	-	-	0.8	0.1	0.1	-	-	8.29 a	0.18 a
	CP	1.8	1.6	2.0	-	1.8	0.8	4.4	1.0	-	1.43 b	15.81 b
	FA	0.6	0.5	0.6	-	0.4	0.5	0.9	0.5	0.3	8.04 a	5.22 a
	FB	0.4	0.6	0.4	0.1	0.2	0.2	1.7	1.5	0.1	7.24 a	7.36 a
	FC	0.5	0.5	0.5	0.1	0.6	0.5	0.7	1.1	0.3	6.95 a	6.76 a
	72 hours											
	CN	2	-	-	-	0.8	0.1	0.1	-	-	8.29 a	0.18 c
	CP	1.8	1.6	2.0	-	1.8	0.8	4.4	1.0	-	1.43 b	15.81 a
	FA	-	0.8	0.8	-	0.3	0.40	0.8	0.8	-	2.3 b	9.35 b
	FB	0.2	0.2	0.2	-	-	-	0.5	-	-	1.5 b	6.13 ab
	FC	0.3	0.6	1.20	-	1.40	1.0	3.0	1.0	-	2.8 b	13.27 b
	Recovery											
	CN	2	-	-	-	0.8	0.1	0.1	-	-	8.29 a	0.18 c
	CP	1.8	1.6	2.0	-	1.8	0.8	4.4	1.0	-	1.43 c	15.81 a
	FA	1.2	0.5	0.2	-	0.4	0.4	0.3	0.7	-	6.13 ab	5.81 b
	FB	0.9	0.6	-	-	0.6	0.1	0.5	0.4	-	4.48 b	6.76 b
	FC	0.6	0.6	-	0.1	0.3	0.1	0.3	0.7	-	3.93 b	6.60 b

A- micronucleus; **B-** anaphase with bridge; **C-** multipolar anaphase; **D-** delayed anaphase; **E-** chromosome breaks; **F-** losses chromosome; **G-** C-metaphase; **H-** adherence; **I-** binucleate cell; **J-** frequency of the mitotic index; **K-** frequency of aberrant cells in relation to dividing cells. Means followed by the same letters did not d iffer at the 5% level of siginificance (mean rank Kruskal -Wallis test).

Table 3 - Chemical analysis of the water (results are reported as ppm) collected at the different sites along the "Córrego dos Bagres" stream in 2001.

		Chemical elements										
		Pb	Fe	Cd	Cr	P	Al	Zn	Cu	Ba	Co	Ni
Summer												
	FA	<0.05	0.21	<0.005	0.05	1.5	0.33	0.11	0.01	0.04	<0.01	<0.01
	FB	<0.025	0.8	<0.005	0.38	2.20	0.43	0.04	0.01	0.05	<0.01	<0.01
	FC	<0.025	0.3	<0.005	0.23	0.11	0.14	0.05	<0.01	0.03	<0.01	<0.01
Autumn												
	FA	<0.025	0.3	<0.005	<0.01	1.5	0.33	0.11	0.01	0.04	<0.01	<0.01
	FB	<0.025	0.5	<0.005	0.11	1,2	0.12	0.05	<0.01	0.04	<0.01	<0.01
	FC	<0.025	0.3	<0.005	0.05	1.1	0.07	0.01	<0.01	0.04	<0.01	<0.01
Winter												
	FA	<0.025	0.04	<0.005	<0.01	1.6	<0.05	0.01	<0.01	0.04	<0.01	0.01
	FB	<0.025	0.18	<0.005	0.02	1.82	0.05	0.02	<0.01	0.04	<0.01	0.01
	FC	<0.025	0.15	<0.005	0.02	1.22	<0.05	0.01	<0.01	0.05	<0.01	<0.01
Spring												
	FA	<0.025	0.16	<0.005	<0.01	<0.1	0.10	0.01	<0.01	0.04	<0.01	<0.01
	FB	<0.025	0.28	<0.005	0.07	0.40	0.06	0.02	<0.01	0.04	<0.01	0.01
	FC	<0.025	0.19	<0.005	0.03	0.22	0.22	0.01	<0.01	0.06	<0.01	0.01

FA- upstream of the effluent disposal site; **FB-** effluent disposal site; **FC-** downtream of the disposal site.

Table 4 - Chemical analysis of the water (results are reported as ppm) collected at the different sites along the "Córrego dos Bagres" stream in 2002.

		Chemical elements										
		Pb	**Fe**	**Cd**	**Cr**	**P**	**Al**	**Zn**	**Cu**	**Ba**	**Co**	**Ni**
Summer												
	FA	<0.025	0.36	<0.005	<0.01	0.32	<0.05	0.01	<0.01	0.05	<0.01	<0.01
	FB	<0.025	0.28	<0.005	<0.01	0.24	0.07	0.01	<0.01	0.05	<0.01	<0.01
	FC	<0.025	0.27	<0.005	<0.01	0.21	0.09	0.01	<0.01	0.05	<0.01	<0.01
Autumn												
	FA	<0.012	0.23	<0.003	<0.005	<0.1	0.05	<0.005	<0.005	0.05	<0.005	<0.005
	FB	<0.012	0.22	<0.003	<0.005	0.11	0.03	0.04	<0.005	0.05	<0.005	<0.005
	FC	<0.012	0.27	<0.003	<0.005	<0.1	0.1	<0.005	<0.005	0.05	<0.005	<0.005
Winter												
	FA	<0.02	0.76	<0.003	0.005	<0.1	0.3	<0.005	<0.005	0.08	<0.005	<0.005
	FB	<0.02	0.46	<0.003	0.01	0.14	0.16	<0.005	<0.005	0.05	<0.005	<0.005
	FC	<0.02	0.19	<0.003	<0.005	<0.1	0.25	<0.005	<0.005	0.01	<0.005	<0.005
Spring												
	FA	<0.02	1.21	<0.003	<0.005	<0.1	2.75	0.01	0.01	0.03	<0.005	<0.005
	FB	<0.02	0.06	<0.003	<0.005	0.21	0.08	0.01	0.005	0.01	<0.005	0.01
	FC	<0.02	1.29	<0.003	<0.005	<0.1	3.48	0.01	0.01	0.01	<0.005	<0.005

FA- upstream of the effluent disposal site; **FB-** effluent disposal site; **FC-** downtream of the disposal site.

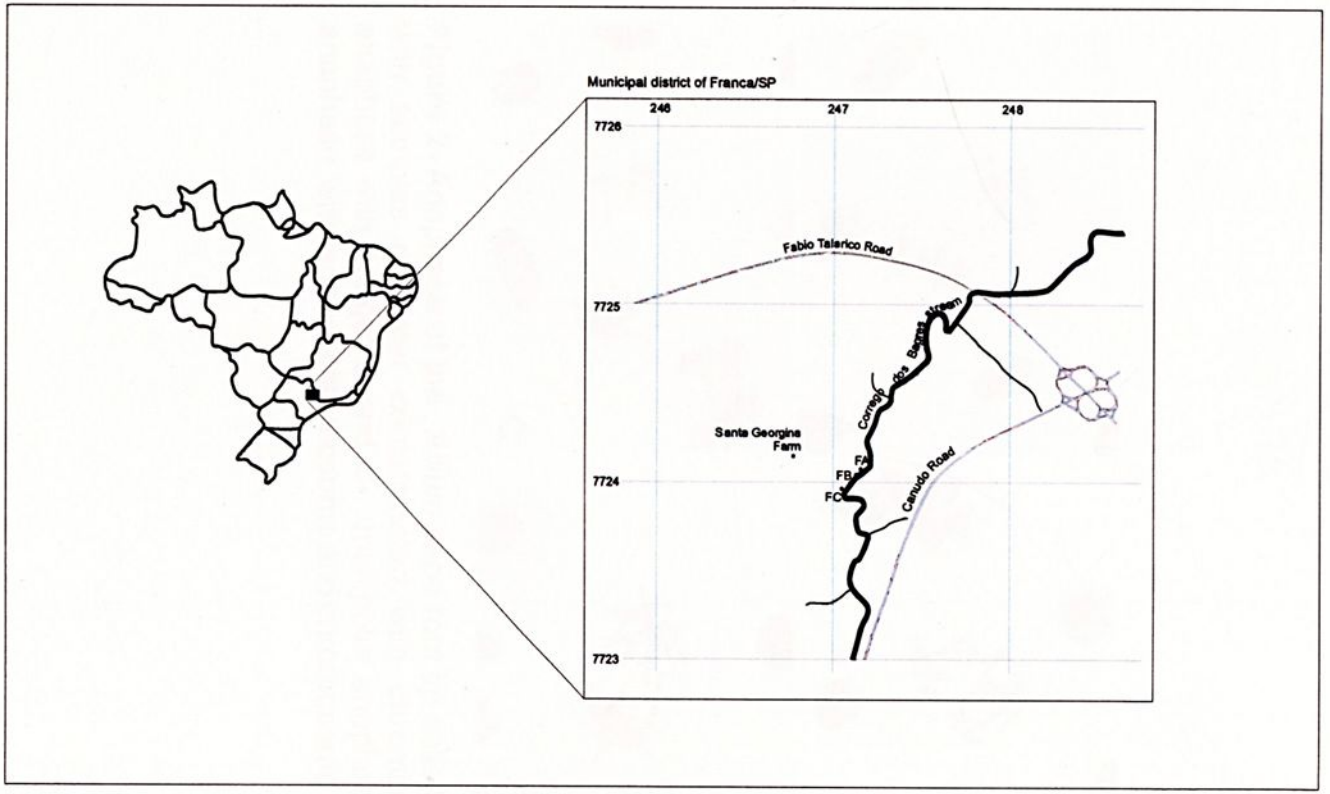

Figure 1 - Geographical localization of the collect site along the "Corrego dos Bagres" stream.

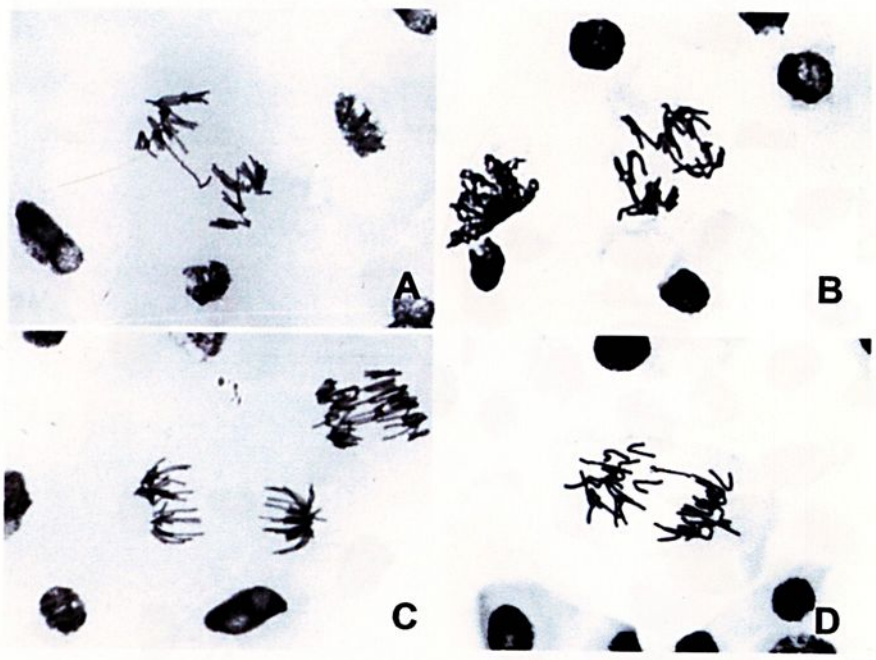

Figure 2- Anaphase of the *Allium cepa* root tip cells treated with samples of water contaminated with chromium: **A-** anaphase with bridge; **B** and **C-** multipolar anaphases; **D-** anaphase with laggard chromosome and chromosome loss.

Figure 3 - *Allium cepa* root tip cells treated with water samples from the Bagres Stream: **A** and **B-**, cells with chromosome breaks showing chromosome fragments; **C-** anaphase with a bridge; **D-** telophase with a bridge; **E-** telophase with a bridge and with chromosomal loss; **F-** telophase with chromosomal loss.

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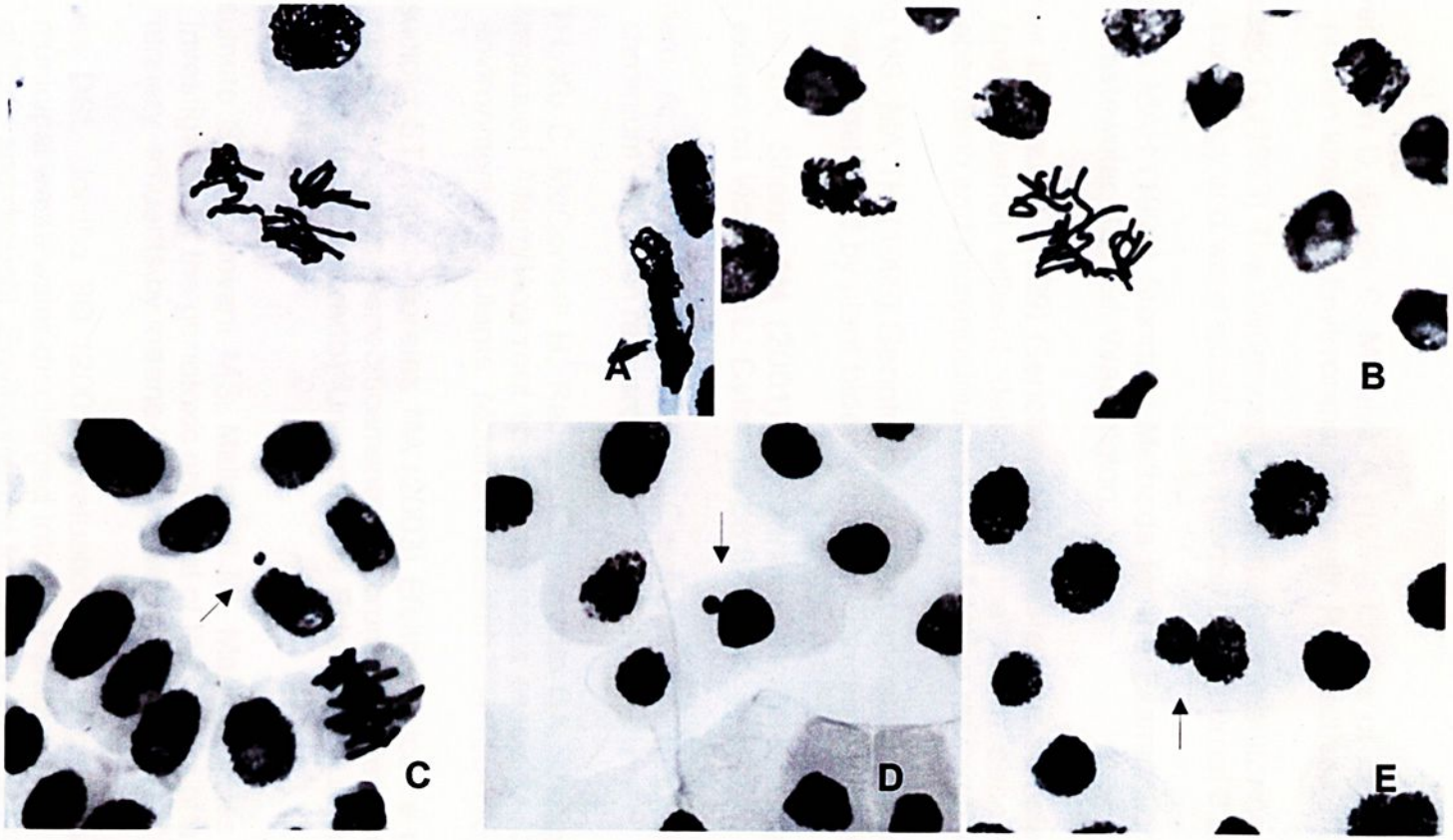

Figure 4 - *Allium cepa* root tip cells treated with water samples from the Bagres Stream contaminated with chromium residues: **A-** chromosome adherence; **B-** C-metaphase; **C** and **D-** cells with micronuclei; **E-** binucleate cell.

Reference

Beyersmann D, Block C, Malviya A (1994) Effects of cadmium on nuclear protein kinase C. Envirronmental Health Perspectives 102:177-180.

Fiskesjö G (1993) The *Allium cepa* in wastewater monitoring. Environmental toxicology and water quality: An International Journal 8:291-298.

Franson MAH (1995) Standard Methods for the Examination of Water and Wastewater. 19th Ed. Washington.

Grover IS, Kaur S (1999) Genotoxicity of wastewater samples from sewage and industrial effluent detected by the *Allium cepa* root anaphase aberration and micronucleus assays. Mutation research 426:183-188.

Kong MS, MA TH (1999) Genotoxicity of contaminated soil and shallow well water detected by plant bioassays. Mutation research 426:221-228.

Horoun SA, Shehri AM (2001) Cytogenetic effects of *Calatropis procera* extract on *Vicia faba* L. Cytologia 66:373-378.

Leonard A, Lauwerys, RR (1980) Carcinogenicity and mutagenicity of chromium. Mutation research 76:227-239.

Ma TH, Xu C, McConnell H, Rabago EV, Arreola GA, Zhang H (1995) The improved *Allium*/*Vicia* root tip micronucleous assay for clastogenicity of environmental pollutants. Mutation research 334:185-195.

Matsumoto ST, Marin-Morales MA (2003) Efeitos tóxicos e genotóxicos de metais pesados, especificamente do cromo trivalente e hexavalente. Monografia (Doutorado) Universidade Estadual Paulista. São José do Rio Preto/SP.

Matsumoto ST, Mantovani MS, Mallaguti MI, Marin-Morales MA (*in press*) Investigation of the genotoxic potential of the waters of a river receiving tannery effluents by means of the *in vitro* comet assay. Cytologia.

Moraes DSL, Jordão BQ (2001) Evaluation of the genotoxic potential of municipal waste water discharged into the Paraguay river during periods of flood and drought. Environmental toxicology 16:113-116.

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Mount DR, Hockett JR (2000) Use of toxicity identification evaluation methods to characterize, identify, and confirm hexavalent chromium toxicity in an industrial effluent. Water research 34:1379-1385.

Nacci D, Nelson S, Nelson W, Jackim E (1992) Application of the DNA alkaline unwinding assay to detect DNA strand breaks in marine bivalves. Marine environmental research 33:83-100.

O'Brien T, Xu J, Patierno SR (2001) Effects of glutathione on chromium-induced DNA crosslinking and DNA polymerase arrest. Molecular and cellular biochemistry 222:173-182.

Patra M, Sharma A (2002) Relative efficacy of *Allium cepa* and *Allium sativum* in anaphase-telophase test screening metal genotoxicity. Biologia 57:409-414.

Rank J, Nielsen MH (1998) Genotoxicity testing of wastewater sludge using the Allium cepa anaphase-telophase chromosome aberration assay. Mutation research 418:113-119.

Sahi AN, Singh SK, Sem PK, Singh RN (1998) Gytogenetic response of hexavalent shromium-induced somatic cell abnormalities in *Allium cepa* Cytobios 96:71-79.

Singh J, Bridgewater LC, Patierno SR (1998) Differential sensitivity of chromium-mediated DNA interstrand crosslinks and DNA-protein crosslinks to disruption by alkali and EDTA. Toxicological sciences 45:72-76.

Smaka-Kincl V, Stegnar P, Lovka M, Toman MJ (1997) The evaluation de waster, surface and ground water quality using the *Allium* test procedure. Mutation Research 368:171-179.

Stohs SJ, Bagchi D (1995) Oxidative mechanisms in the toxicity of metal ions. Free radical biology and medicine 18:321-336.

Sudhakar D, Panda KK, Panda BB (1998) Biomonitoring of low levels of mercurial derivatives in water and soil by *Allium* micronucleus assay. Mutation research 203:11-23.

Sudhakar R, Ninge-Gowda KN, Venu G (2001) Mitotic abnormalities induced by silk dyeing industry effluents in the cells of *Allium cepa*. Cytologia 66:235-239.

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Von Burg R, Liu D (1993) Chromium and hexavalent chromium. Journal of applied toxicology 13:225-230.

Zhitkovich A, Voitkun V, Costa M (1996) Formation of the amino acid-DNA complexes by hexavalent and trivalent chromium *in vitro*: Importance of trivalent chromium and the phosphate group. Biochemistry 35:7275-7282.

6. – ARTIGO 04

ASSESSMENT OF THE GENOTOXIC EFFECT OF CHROMIUM RESIDUES PRESENT IN TANNERY EFFLUENTS USING THE MICRONUCLEUS TEST AND COMET ASSAY

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Abstract

Analysis of the cytotoxic effects of heavy metals has received special attention due to the fact that these metals are potentially mutagenic and induce the formation of tumors in experimental organisms and in humans exposed to them. The objective of the present study was to evaluate the genotoxic potential of water resources that receive tannery effluents and, as a consequence, are possibly contaminated with chromium. Three sites along the "Córrego dos Bagres" stream, municipality of Franca/SP, Brazil, were analyzed using the comet assay and the micronucleus test performed on fish (*Oreochromis niloticus*) erythrocytes. Nuclear abnormalities observed in the erythrocytes were blebbed, notched and lobed nuclei, which are probably the result of the genotoxic effect of chromium residues present in the water of the river studied. In the comet assay, the highest frequency of damage was observed for the samples obtained from the site of effluent disposal, thus confirming the genotoxic effect of chromium residues.

Key words: Micronucleus, Nuclear abnormality, Genotoxicity, Comet assay, Chromium

Introduction

Pollution of water resources is a serious and growing problem. Despite the existence of relevant legislation, pollution of the aquatic environment by toxic chemical pollutants continues to occur. Domestic and industrial effluents are the main sources responsible for the contamination of aquatic environments (Claxton *et al.*, 1998; White and Rasmussen, 1998).

Aquatic organisms such as fish accumulate pollutants deriving directly from the contaminated water or indirectly through the ingestion of contaminated aquatic organisms. Thus, genotoxic pollutants may lead to the contamination not only of addition to the aquatic organisms themselves, but of the entire ecosystem and, finally, of humans through the food chain.

Model systems such as fish are important to determine the distribution and toxic effects of chemical contaminants present in the aquatic environment (Al-Sabti and Metcalfe, 1995). A variety of teleost species have been used for the study of the mutagenic, clastogenic and teratogenic effects of environmental contaminants during the first stages of life. Metcalfe (1988) used some fish species (*Oncorthychus mykiss* and *Oryzias latipes*) as a test organism by for the study of carcinogenesis.

According to Al-Sabti and Metcalfe (1995) and Dashwood and Bariley (1998), *in vivo* techniques such as the micronucleus test performed in fish test systems have been shown to be efficient for the assessment of the genotoxic potential and water quality monitoring.

The analysis of the frequency of micronuclei during interphase represents a rapid and easy technique. The micronucleus test in fish erythrocytes has been used as a first measure in the evaluation of clastogenic potential. Since teleost erythrocytes possess a nucleus, they are a good tool for the detection of clastogenic substances in water. Several studies have shown a high incidence of micronuclei in fish peripheral erythrocytes after exposure to different pollutants under both field and laboratory conditions (Al-Sabti, 1986; Hose *et al.*, 1987; Metcalfe, 1988; Minissi *et al.*, 1996).

The comet assay or SCGE (single cell gel electrophoresis assay) is a genotoxicity test able to detect DNA damage induced by alkylating, intercalating and oxidizing agents (Kosz-Vnenchak and Rokosz, 1997; Tice *et al.*, 2000).

The comet assay has also been used as an important tool to monitor the genotoxic effect of substances on aquatic environments. For this purpose, fish are used as a test organism in which it is possible to detect DNA damage induced by direct mutagens and pro-mutagens dissolved in water, or even by ocean water samples (Mitchelmore and Chipman, 1998). This technique has also been employed in the determination of the genotoxic potential of water resources such as rivers and lakes.

Contamination of the environment by heavy metals has been increasing every year (Majer *et al.*, 2002). Analysis of the cytotoxic effects of

heavy metals has received special attention due to the fact that these metals are potentially mutagenic and induce the formation of tumors in experimental organisms and humans exposed to them (Garcia-Rodríguez *et al.*, 2001).

The genotoxic effects of mercury, methylmercury, selenium, as well as a mixture of these substances, have been evaluated by Al-Sabti (1991) in studies on Prussian carp erythrocytes. The author observed a high frequency of micronuclei in the cells when compared to the control group.

According to Carrasco *et al.* (1990), nuclear abnormalities have been described and classified as blebbed, notched and lobed nuclei. These abnormalities have also been reported by several other investigators, but were simply called genotoxic damage (Pachecco and Santos, 1998; Ayylon and Garcia-Vazquez, 2000; Bombail *et al.*, 2001).

Nuclear abnormalities are frequently observed in fish erythrocytes. Eiras (1990) identified nuclear abnormalities in fish with folic acid deficiency, fish affected by viral necrosis, as well as in specimens exposed to chlorine and cadmium.

Chromium is considered to be a metal which is toxic, genotoxic and carcinogenic to man and animals (Von Burg and Liu, 1993). Cells or test organisms exposed to chromium VI show different types of damage in the DNA molecule such as DNA strand exchange, DNA-protein crosslinks, base oxidative damage, and DNA strand breaks (Rojas *et al.*, 1999).

Trivalent III chromium is used for leather tanning. Although chromium III is considered to be mutagenic, it does not pose immediate danger to the cell since in this state the metal is unable to cross the cell membrane, a fact that impairs easy access to the cell. Chromium III, however, may be transformed in the environment into hexavalent (VI) chromium, a stage to which the cell membrane is highly permeable.

According to Sugiyama (1992), cells are permeable to chromate – hexavalent chromium (VI) through the sulfate transport system. Inside the cell, chromium VI is reduced to the trivalent form III and this reduced form induces genotoxic effects in the organism (Bianchi *et al.*, 1983).

Al-Sabti (1994) determined the cytological effects of chromium VI and chromium III in erythrocytes of *Carassus auratus gibelio* fish by means of the micronucleus test. Fish exposed to sublethal chromium concentrations in the laboratory and fish collected from chromium-contaminated rivers showed a significant frequency of micronuclei when compared to the negative control, thus demonstrating the true genotoxic effect of chromium.

The present study determined the genotoxic potential of water resources that receive tannery effluents and, as a consequence, are possibly contamined with chromium. Three sites along the "Córrego dos Bagres" stream, municipality of Franca, São Paulo, were studied using the comet assay and the micronucleus test in fish erythrocytes (*Oreochromis niloticus*).

cm 1 2 3 4 5 6 7 8 unesp 12 13 14 15 16 17 18

Material and Methods

Physicochemical analysis of the water

For the determination of pH, conductivity and cations, the water samples were first filtered through qualitative filter paper. The analyses were based on the Standard Methods for the Examination of Water and Wastewater (Franson, 1995).

For cation determination, the samples were first acidified with HNO_3, pH 1, and the cations were analyzed sequentially by inductively coupled plasma atomic emission spectrometry (ICP-AES) with ultrasonic nebulization. The following elements were determined: calcium, magnesium, strontium, silicon, iron, manganese, aluminum, zinc, chromium, cobalt, nickel, lead, cadmium, phosphorus, copper, and barium.

The standard solutions used to construct the calibration curves for the elements analyzed in 0.1% HNO_3 medium were obtained by appropriate dilutions of the standard solutions of 1000 ppm Titrisol (Merck).

Bioassay

Water samples were collected at three different sites along the "Córrego dos Bagres" stream: 500 m upstream of the tannery effluent (FA), at the site of disposal of the tannery effluent (FB), and 300 m downstream of the tannery effluent site (FC) (Figure 1).

cm 1 2 3 4 5 6 7 8 unesp 12 13 14 15 16 17 18

The water samples collected from the three sites were placed in aquaria at a dilution of 1:1 with well water under constant aeration for 3 days. After this period, 6 fish (*Oreochromis niloticus*) were added to each aquarium and left in this mixture for 72 h. The fish, with a mean size of 10 cm, were kindly provided by the Fish Farm of the State University of Londrina, Paraná.

Comet assay

Blood samples were obtained from the fish with a heparinized syringe. A 10-µl blood sample was diluted in 1000 µl fetal bovine serum. The slides were mounted with 10 µl of the cell suspension + 120 µl of 0.5% low melting point agarose at 37°C. The slides were kept in lysis buffer (1 ml Triton X-100, 10 ml DMSO and 89 ml lysis stock solution, pH 10.0 – stock solution: 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, ~8.0 g solid NaOH, 10 g sodium lauryl sarcosinate in 1 liter) in a refrigerator for 1 h. After lysis, the slides were incubated in 300 mM NaOH + 1 mM EDTA buffer, pH >13, for 20 min for DNA denaturation, and submitted to electrophoresis at 25 V and 300 mA for 20 min. The slides were then neutralized with 0.4 M Tris for 15 min and fixed in ethanol for 10 min.

A total of 100 nuclei were analyzed per blood sample. The slides were stained with ethidium bromide (0.02 mg/ml) and analyzed under a Nikon fluorescence microscope equipped with a B-3^{A} filter (excitation: λ = 420-490

nm, emission barrieir: λ = 520 nm) and a 40x lens. The nuclei were classified visually according to fragment migration into: class 0 (no damage), class 1 (small damage), class 2 (medium damage), and class 3 (large damage). The χ^2 test was used for statistical analysis, in which the total number of altered nuclei observed for each treatment was compared to the results obtained for the negative control.

Micronucleus test

Blood samples were obtained by tail puncture using heparinized syringes. Smears were prepared on slides, with 6 slides being obtained for each previously determined study site. After 24 h, the material was fixed in absolute methanol for 10 min and stained with 5% Giemsa for 20 min. The number of erythrocytes with micronuclei was determined by analysis of 2000 cells per fish as described by Huber (1992). The Kruskal-Wallis test was used to compare the number of normal cells and cells with a micronucleus obtained for each collection site with the results observed for the negative control.

Results and Discussion

Both the comet assay and the micronucleus test using the fish *O. niloticus* as test organism showed that the water collected at the three sites

along the "Córrego dos Brages" stream was significantly genotoxic when compared to the negative control.

The highest frequency of micronuclei and nuclear abnormalities was observed for the FB site (0.45 and 2.53%, respectively) (Table 1 and Figures 2 and 3). The data indicate, as expected, that the water from the FB site is more contaminated due to the disposal of tannery wastewater, leading to a higher rate of abnormalities. These data were also confirmed by chemical analysis of the water collected from this site (Table 2). Our results agree with those reported by Von Burg and Liu (1993) and Blasiak and Kowalik (2000), who proposed that chromium exerts a genotoxic effect on animals due to its potential to promote different damage to the DNA molecule.

A smaller genotoxic effect was observed upon the analysis of micronuclei and nuclear abnormalities for the sites upstream (FA) and downstream (FC) of the effluent compared to the results obtained for the site of effluent disposal. At the FA site, the frequency of micronuclei and nuclear abnormalities was 0.20 and 1.77%, respectively. The frequency of micronuclei at this site was not significant compared to the negative control. At the FC site, the frequency of micronuclei and nuclear abnormalities was 0.26 and 2.12%, respectively (Table 1 and Figures 2 and 3). The frequency of micronuclei was significant at the FB and FC sites compared to the FA site and the negative control, demonstrating a smaller genotoxic effect upstream of the river (FA site) than that observed at the site of effluent disposal (FB

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site) and below the disposal site. These data confirm that chromium residues derived from leather tanning activities in the Franca/SP region represents a polluting agent with genotoxic effects.The nuclear abnormalities observed in erythrocytes were classified as blebbed, notched and lobed nuclei according to the description and classification of Carrasco *et al.* (1990), Ayylon and Garcia-Vazquez, (2000) and Çavas and Ergene-Gözükara (2003), as shown in Figure 3. The FB and FC sites showed a significant frequency of nuclear abnormalities compared to the negative control, demonstrating that these abnormalities are the consequence of the genotoxic effect of chromium residues present in the river that receives tannery wastewater.

Analysis of the comet assay results revealed significant genotoxic effects for the FB and FC sites compared to the negative control (Table 3 e Figure 4). The highest frequency of damage was observed for the FB site, confirming the genotoxic effect of the chromium residues present at the site that receives the tannery effluent. The comet assay has been shown to be efficient in the detection of DNA damage provoked by genotoxic agents present in aquatic environments. Mitchelmore and Chipman (1998) stated that the comet assay using fish as the test organism is an effective technique for monitoring the genotoxic potential of aquatic environments.

The damage detected by the comet assay for the site upstream of the effluent was not significant compared to the negative control (Table 3 e Figure 4). This finding can be explained by the fact that this region of the

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stream has not yet suffered the impact caused by the chromium residue contamination resulting from the emission of tannery wastewater.

The legislation of the State of São Paulo establishes a maximum chromium concentration of the order of 0.05 mg/L for the emission of industrial effluents into rivers. In the present study, a genotoxic effect on fish erythrocytes was only observed for sites where the total chromium concentration was above the value established by the legislation (0.05 mg/L). However, Matsumoto *et al.* (in press), using the comet assay and CHO-K1 cell cultures, found a genotoxic effect for water contaminated with tannery effluent containing a chromium concentration lower than that permitted by the legislation of the State of São Paulo (0.01 mg/L). These results confirm the genotoxic potential of this heavy metal.

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Table 1 - frequency of normal cells, cells with nuclear abnormalities and cell with a micronucleus obtained from the different collected site.

Treatment	Total cells			
	Analised	Normal cells	nuclear abnormalities	MN
	2.000	1980	20	0
	2.000	1983	15	2
NC	2.000	1976	23	1
	2.000	1984	15	1
	2.000	1991	9	0
	2.000	1956	44	0
Total	**6.000**	**11870**	**126**	**4**
Frequency (%)		**98.92**	**1.05**	**0.03**
	2.000	1956	38	6
	2.000	1922	71	7
Site FA	2.000	1991	8	1
	2.000	1985	11	4
	2.000	1946	51	3
	2.000	1964	33	3
Total	**6.000**	**11764**	**212**	**24**
Frequency (%)		**98.03**	**1.77**	**0.2**
	2.000	1956	36	8
Site FB	2.000	1913	74	13
	2.000	1938	53	9
	2.000	1966	30	3
	2.000	1935	56	9
	2.000	1937	55	12
Total	**6.000**	**11645**	**304**	**54**
Frequency (%)		**97.02**	**2.53**	**0.45**
	2.000	1959	35	6
	2.000	1947	48	5
Site FC	2.000	1956	39	5
	2.000	1951	42	7
	2.000	1946	50	4
	2.000	1955	40	5
Total	**6.000**	**11714**	**254**	**32**
Frequency (%)		**97.62**	**2.12**	**0.26**

NC- negative controle; FA- upstrem of the effluent disposal site; FB- effluent disposal site; FC- downstream of the effluent disposal site.

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Table 2 - Chemical analysis of the water (results are reported as ppm) collected at the different sites along the "Corrego dos Bagres" stream, Franca/SP.

Collection site	Chemical elements															
	Si	**Ca**	**Sr**	**Mg**	**Pb**	**Fe**	**Mn**	**Cr**	**P**	**Al**	**Zn**	**Cu**	**Ba**	**Co**	**Ni**	**Cd**
FA	5.2	50.0	0.25	16.8	<0.05	0.21	0.07	0.05	1.5	0.33	0.11	0.01	0.04	<0.01	<0.01	<0.005
FB	6.0	20.7	0.12	5.5	<0.025	0.8	0.10	0.38	2.20	0.43	0.04	0.01	0.05	<0.01	<0.01	<0.005
FC	6.2	15.3	0.08	3.3	<0.025	0.3	0.04	0.23	0.11	0.14	0.05	<0.01	0.03	<0.01	<0.01	<0.005

FA- upstrem of the effluent disposal site; FB- effluent disposal site; FC- downstream of the effluent disposal site.

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Table 3- Analysis of the changes observed in erythrocytes (*Oreochromis niloticus*) treated with waters collected at distinct points along the stream which receives tannery effluents in the region of Franca/SP.

Treatment	Total cells		Class				Escores
	analised	with comet	0	1	2	3	
NC	100	26	74	22	4	0	30
	100	21	79	17	4	0	25
	100	20	80	19	1	0	21
	100	18	82	14	3	1	23
	100	77	23	57	16	4	101
	100	19	81	14	5	0	24
Total	**600**	**181**	**419**	**143**	**33**	**5**	**X = 37,33**
FA	100	19	81	10	8	1	29
	100	15	85	8	4	3	25
	100	18	82	17	1	0	19
	100	42	59	29	9	3	56
	100	47	53	32	9	6	68
	100	54	46	42	9	3	69
Total	**600**	**195**	**406**	**138**	**40**	**16**	**X = 44,33**
FB	100	41	59	34	6	1	49
	100	21	79	13	4	4	33
	100	59	41	48	7	4	74
	100	39	61	30	4	5	53
	100	57	43	41	10	6	79
	100	76	24	34	27	15	133
Total	**600**	**293*, ****	**307**	**200**	**58**	**35**	**X = 70,17**
FC	100	47	53	35	7	5	64
	100	53	47	31	15	7	82
	100	53	47	37	8	8	77
	100	48	52	21	15	12	87
	100	23	77	15	3	5	36
	100	20	80	14	6	0	26
Total	**600**	**244*, *****	**356**	**152**	**54**	**37**	**X = 62,0**

NC- negative control; FA- upstream of the effluent disposal site; FB- effluent disposal site; FC- downstream of the effluent disposal site. *significant statistic difference in relation to the NC; ** significant statistic difference in relation to the site A; *** significant statistic difference in relation to the site B.

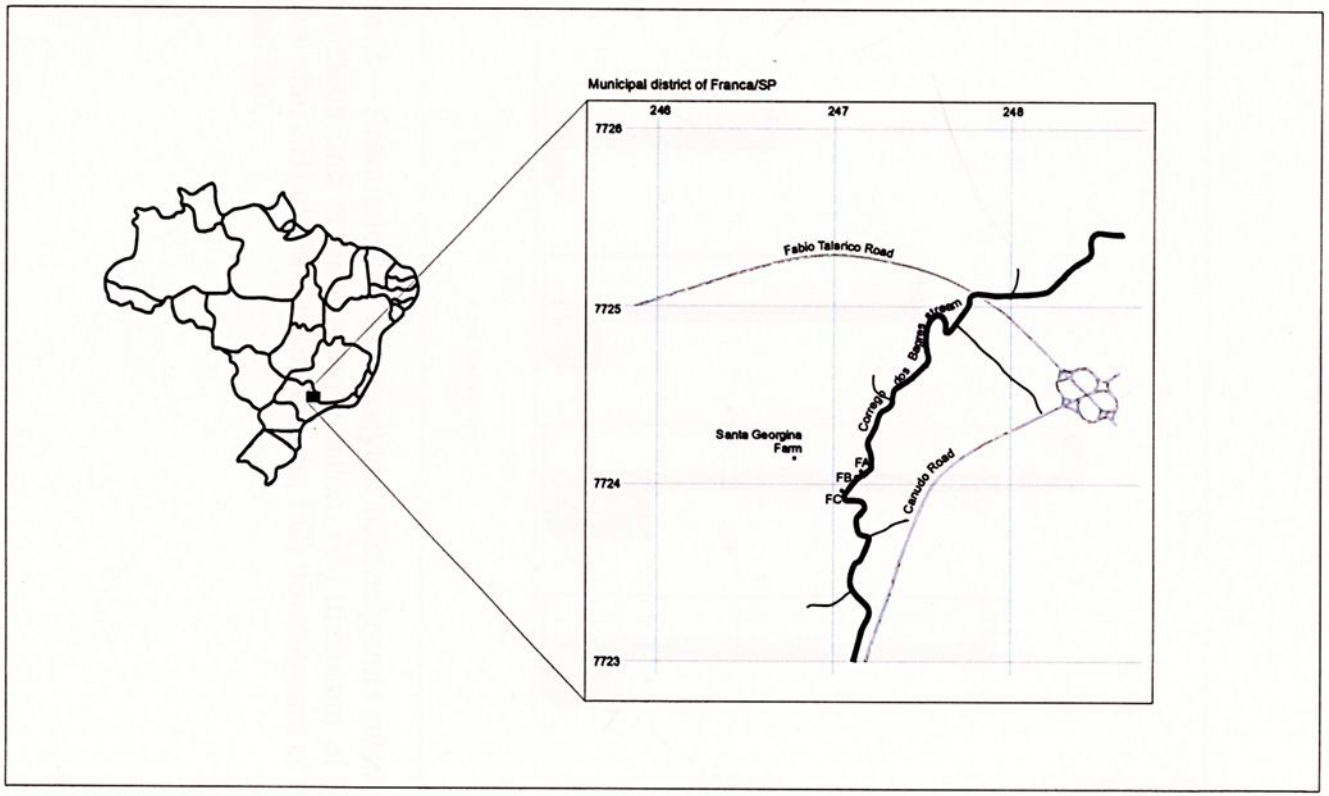

Figure 1 - Geographical localization of the collect site along the "Corrego dos Bagres" stream.

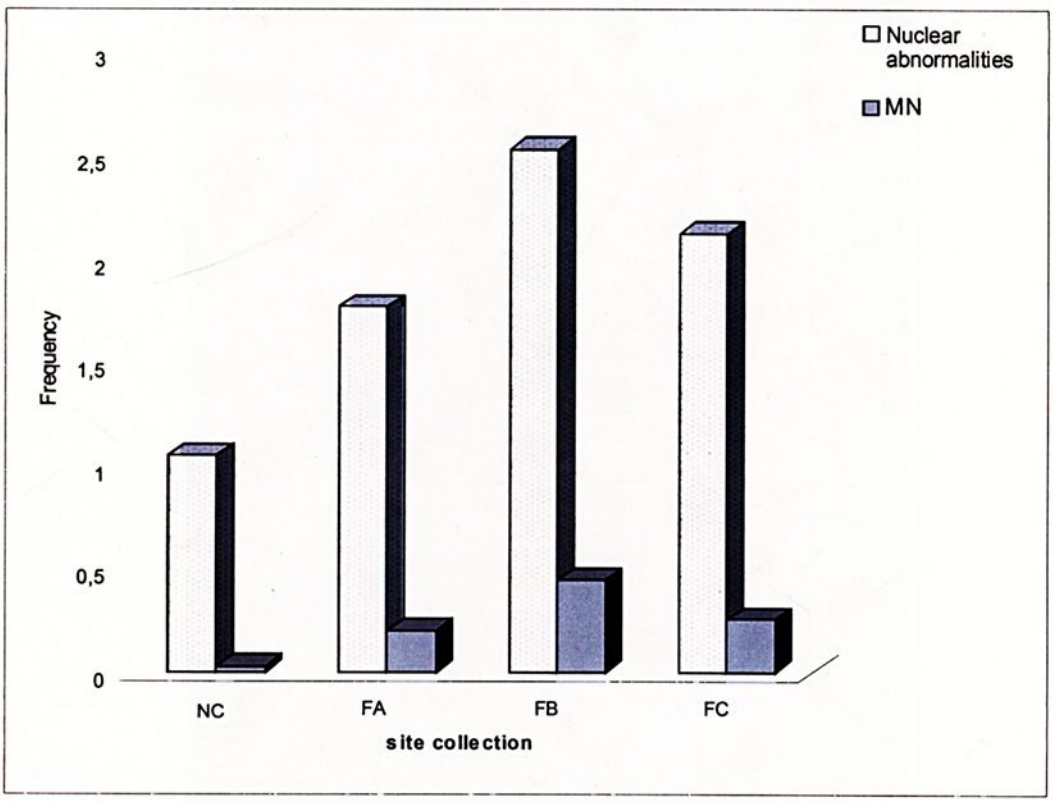

Figure 2 – Frequency of erythrocytes (*Oreochromis niloticus*) with a micronucleus and nuclear abnormalities. FA) upstream of the effluent disposal site; FB) effluent disposal site, FC) downstream of the effluent disposal site.

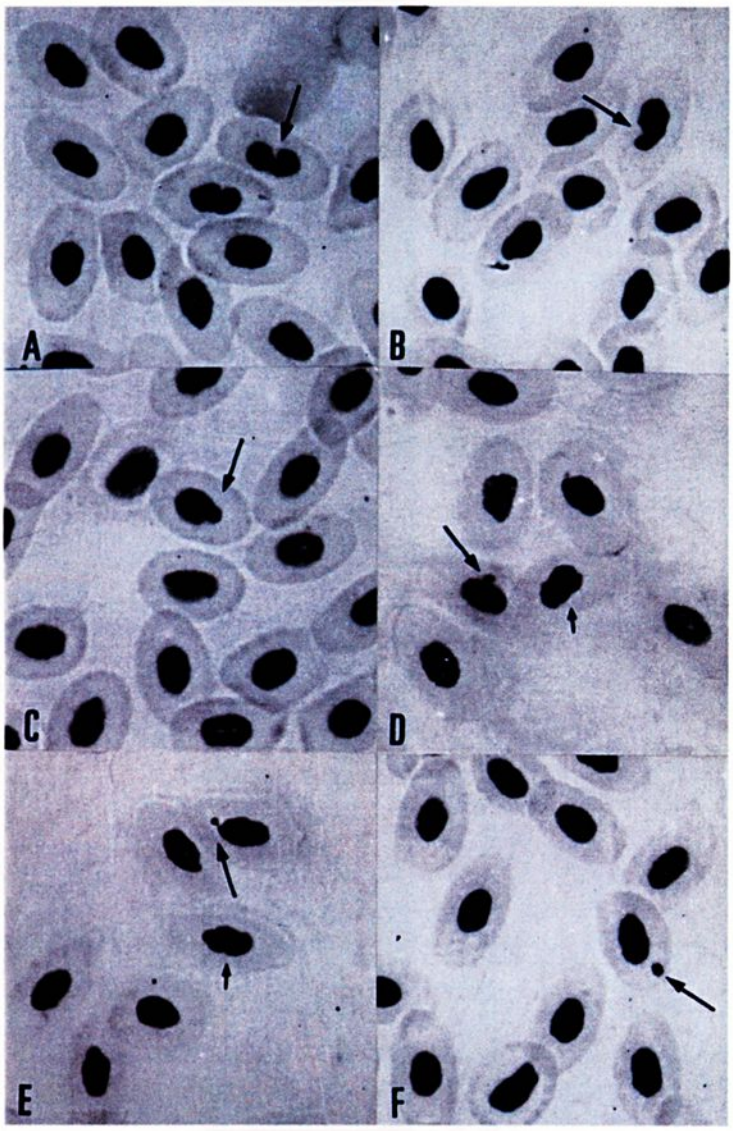

Figure 3 – Erytrocytes (*Oreochromis niloticus*) with a micronucleus and nuclear abnormalities: **A** and **B**-Notched nuclei; **C**-Lobed nuclei; **D**-Lobed nuclei (large arrow) and blebbed nuclei (small arrow); **E**-Broken eggs (large arrow) and blebbed nuclei (small arrow); F-Micronucleus

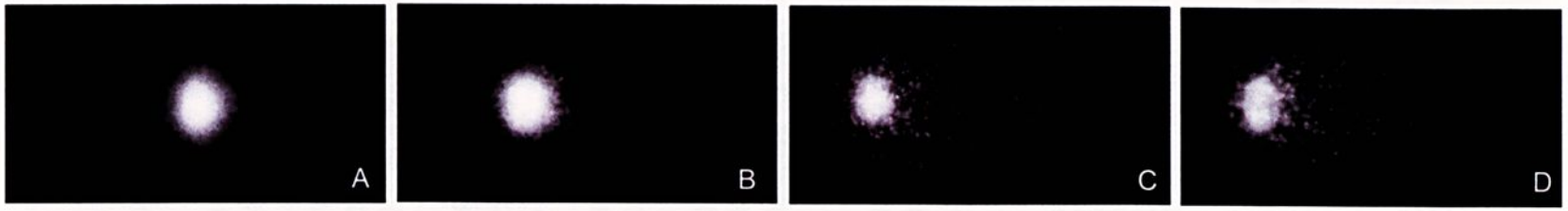

Figure 4 - Results of the comet assay performed with *Oreochromis niloticus* erythrocytes. **A**- Class 0 (cell without damage); **B**-Class 1 (small damage); **C**- Class 2 (medium damage) and **D**- Class 3 (large damage).

References

Al-Sabti K (1986) Clastogenic effects of five carcinogenic chemicals on the cells of the common *Cyprinus carpio L.* Comp. Biochem Physiol 85:5-9.

Al-Sabti K (1991) Micronuclei induced by selenium, mercury, methylmercury and their mixtures in binucleated blocked fish erythrocyte cells. Mutat Res 320: 157-163.

Al-Sabti K (1994) Chromium-induced micronuclei in fish. J Appl Toxicol 14:333-336.

Al-Sabti K and Metcalfe C (1995) Fish micronuclei for assessing genotoxicity in water. Mutat Res 343: 121-135.

Ayylon F and Garcia-Vazquez G (2000) Induction of micronuclei and other nuclear abnormalities in European minow *Phoxinus phoxinus* and mollie *Paecilia latipinna*: an assessment of the fish micronucleus test. Mutat Res 467: 177-186.

Bianchi V, Celotti L, Lanfreanchi G, Majone F, Marin G, Montaldi A, Sponza G, Tamino G, Venier P, Zantedeschi A and Levis AG (1983) Genetic effects of chromium compounds. Mutat Res 117: 279-300.

Blasiak J and Kowalik J (2000) A comparison of the in vitro genotoxicity of tri- and hexavalent chromium. Mutat Res 469: 135-145.

Bombail V, Gordon E and Batty J (2001) Application of the comet and micronucleus assays to butterfish (*Pholis gunnelus*) erythrocytes from the firth of forth, Scotland. Chemosphere 44: 283-392.

Carrasco KR, Tilbury KL and Mayers MS (1990) Assessment of the piscine micronuclei test as an *in situ* biological indicator of chemical contaminants effects. Can J Fish Aquat Sci 47: 2123-2136.

Claxton LD, Houk VS and Hugles TJ (1998) Genotoxicity of industrial wastes and effluents. Mutat Res 410: 237-243.

Çavas T and Ergene-Gözükara S (2003) Micronuclei, nuclear lesions and interphase silver-stained nucleolar organizer regions (AgNORs) as

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cyto-genotoxicity indicators in *Oreochromis niloticus* exposed to textile mill effluent. Mutat Res 534: 93-99.

Dashwood RH And Bariley GS (1998) Use of fish and fish transgenics in laboratory and field genotoxicology studies. Mutat Res 399: 123-124.

Eiras JC (1990) Observations on erythrocytes abnormalities in fish. Bull Eur Ass Fish Pathol 10: 64-67.

Franson, M.A.H. (1995). Standard Methods for the Examination of Water and Wastewater. 19th Ed.. Washington.

Garcia-Rodríguez MC, López-Santiago V and Altamirano-Lozano M (2001) Effect of chlorophyllin on chromium trioxide-induced micronuclei in polychromatic erythrocytes in mouse peripheral blood. Mutat Res 496: 145-151.

Hose JE, Cross JN, Smith SG and Diehl D (1987) Elevated circulating erythrocyte micronuclei in fishes from contaminated sites of Sourthern California. Mar Environ Res 22: 167-176.

Huber VS (1992) The genotoxicity of industrial wastes and effuents. Mutat Res 277: 91-138.

Kosz-Vnenchak M and Rokosz K (1997) The "Comet" assay for detection of Potential genotoxicity of polluted water. Folia boil 45: 153-239.

Majer BJ, Tscherko D, Paschke A, Wennrich R, Kundi M, Kandeler E and Knasmüller S (2002) Effects of heavy metal contamination of soils on micronucleus indusction in *Tradescantia* and on microbial enzyme activities: a comparative investigation. Mutat. Res. 515:111-124.

Matsumoto ST, Mantovani MS, Mallaguti MI and Marin-Morales MA (*in press*) Investigation of the genotoxic potential of the waters of a river receiving tannery effluents by means of the *in vitro* comet assay. Cytologia.

Metcalfe CD (1988) Induction of micronuclei and nuclear abnormalities in the erythrocytes of mudminnows and brow bullheads. Bull Environ Contam Toxicol 40: 489-495.

Minissi S, Ciccotti E and Rizzoni M (1996) Micronucleus test in erythrocytes of *Barbus plebejus* (Teleostei, Pisces) from two natural environments:

unesp

a bioassay for the ub suty detectuib if nytagebs ub fresgwater, Mutat Res 367: 245-251.

Mitchelmore CL and Chipman JK (1998) DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. Mutat Res 399: 135-147.

Pachecco M and Santos MA (1998) Induction of liver eirod and erythrocytic nuclear abnormalities by cyclophosphamide and PAHs in *Anguilla anguilla* L. Ecotoxicol Environ Saf 40: 71-76.

Rojas E, Herrera LA, Poirier LA and Ostrosky-Wegman P (1999) Are metals dietary carcinogens? Mutat Res 443: 157-181.

Sugiyama M (1992) Role of physiological antioxidants in chromium (VI)-induced cellular injury. Free Radic Biol Med 12: 397-407.

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC and Sasaki YF (2000) Single Cell Gel/Comet Assay: Guidelines for *in vitro* and *in vivo* Genetic Toxicology Testing. Environ Mol Mutag 35: 206-221.

Von Burg R and Liu D (1993) Chromium and hexavalent chromium. J Appl Toxicol 13: 225-230.

White PA and Rasmussen (1998). The genotoxic hazards of domestic wastes in surface waters. Mutat Res 410: 223-236.

7. – ARTIGO 05

INVESTIGATION OF THE GENOTOXIC POTENTIAL OF THE WATERS OF A RIVER RECEIVING TANNERY EFFLUENTS BY MEANS OF THE *in vitro* COMET ASSAY

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Summary

The comet assay has been described as an efficient tool for the detection of changes in the DNA molecule of cells exposed to contaminating agents *in vivo* and *in vitro*. The possible environmental contamination due to the persistence of chromium residues from tannery effluents was determined in the waters of the "Córrego dos Bagres" stream, Municipal district of Franca/SP, by the comet assay on CHO-K1 cells. Water samples were collected during the four seasons of the year 2001 at three distinct stations along the river. The data suggest that the comet test showed good sensitivity for the environmental monitoring of these waters and indicated that this test can be efficient for the determination of the quality of waters contaminated with effluents containing heavy metal residues such as chromium.

Key words Comet assay, chromium, genotoxicity, CHO-K1.

Introduction

Industrialization and urbanization processes have turned the question of environmental contamination increasingly critical. The contamination of aquatic ecosystems with substances originating from industrial wastes is a matter that is not always treated with the importance it deserves, despite the concern raised by the harmful effects of these substances, with a consequent alteration of continental water quality (White and Rasmussen, 1998).

According to Mitchelmore and Chipman (1998), the comet assay is a methodology that can be potentially used to investigate breaks in the DNA strand, which can serve as a bioindicator of genotoxicity in fish and in other aquatic organisms. These biomarkers are important for the assessment of carcinogenic potential, of the effect on the reproductive system of organisms and of other adverse effects of pollution.

Recently, the comet assay has been used as an effective tool for environmental biomonitoring, permitting the detection of changes in the DNA molecule of cells exposed to chemical and physical agents *in vitro* or *in vivo* (Darroudi and Natarajan, 1993; McNamee et al. 2000; Tice et al. 2000). It is a sensitive and rapid method for measuring DNA damage and repair in individual cells, with damage being detected by electrophoretic microgel migration (Olive and Banáth, 1993; Klaude et al. 1996; Mitchelmore and Chipman, 1998).

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An important study conducted by Pandrangi et al. (1995) showed that the comet assay was efficient in assessing DNA damage provoked by heavy metal pollution in sediments of the Great Lakes (Canada). Nacci et al. (1992) reported a reduction in DNA strand breaks when organisms previously exposed to sediments contaminated with heavy metals were exposed to an environment with controlled conditions (laboratory) without the presence of these pollutants. According to these investigators, this response reflects reversibility rather than persistence of damage after normalization of the environmental conditions.

Experimentally tested hexavalent chromium components proved to be potentially carcinogenic to man and animals since they induced cell transformations *in vitro*, causing various cytogenetic alterations in different biological systems such as point mutations, physicochemical changes in nucleic acids, chromosomal aberrations, and changes in the repair mechanism, among others (Levis et al. 1978).

The objective of the present study was to assess the possible environmental contamination derived from the persistence of chromium residues in tannery effluents dumped into the waters of the "Córrego dos Bagres" stream, Municipal district of Franca/SP, using the comet assay applied to CHO-K1 cells.

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Materials and methods

Sample collect

Water collects were performed during the four seasons of the year 2001 at three different stations along the "Córrego dos Bagres" stream, Municipal district of Franca/SP. The collect sites selected were: before the site of tannery effluent dumping (200 m upstream) – FA, dumping site – FB, and after the dumping site (200 m downstream) – FC (Figure 1). At each collect, 2 liters of water were obtained at a depth of 30 cm according to routine criteria for chemical analyses. The water samples were placed in plastic bottles and carried in an insulated box with ice to Universidade Estadual Paulista – UNESP – Rio Claro /SP.

Physicochemical analysis of the water

The analyses were based on the Standard Methods for the Examination of Water and Wastewater (Franson, 1995).

For cation determination, the samples were first acidified with HNO_3, pH 1, and the cations were analysed sequencialy by inductively coupled plasma atomic emission spectrometry (ICP-AES) with ultrasonic nebulization. The following elements were determined: calcium, magnesium, strontium, silicon, iron, manganese, aluminium, zinc, chromium, cobalt, nickel, lead, cadmium, phosphorus, copper, and barium.

The standard solutions used to construct the calibration curves for the elements analyzed in 0.1% HNO_3 medium were obtained by appropriate dilutions of the standard solutions of 1000 ppm Titrisol (Merck).

Culture conditions and treatments

CHO-K1 cells were cultured in D-Mem + HAMF10 (1:1) medium supplemented with 10% Bovine Serum Albumin at 37ºC. For the treatments a concentrated culture medium (stock) was prepared, to be diluted 3:1 with the water samples collected. The water samples collected at each station were added to the concentrated culture medium at the treatment time.

After 24 hours of culture in normal medium, CHO-K1 cells were kept for 2 hours (37ºC) in the mixture of medium and water collected from the stream, whose pH was adjusted to 7.2 before treatment. The positive control was treated with methylmethanesulfonate (MMS) at the concentration of 10^{-4}M and the negative control was only kept in culture medium. Three independent experiments were performed.

Comet assay

The slides for microscope observation were mounted with 20 µl of the above cellular suspension and 120 µl of low melting point agarose at 37ºC. The slides were bathed in a lysis solution (1 ml of Triton X-100, 10 ml of DMSO

and 89 ml of lysis stock solution [2.5 M NaCl, 100 mM EDTA, 10mM Tris, pH 10, ~8 g solid NaOH, and 10 g lauryl sarcosinate, pH 10]), in the dark, in a refrigerator at about 8ºC for 1 hour.

After lysis, the slides were placed in buffer containing 0.3 N NaOH and 1 mM EDTA, pH>13, for 20 minutes for DNA unwinding. Electrophoresis was performed for 20 minutes at 25 V, 300 mA (~1.0 V/cm) and the slides were them neutralized for 15 minutes in 0.4 M Tris, fixed in absolute ethanol for 10 minutes, and stained with ethidium bromide (0.02 µg/ml).

Comet assay analysis

The comet observations were performed at 400X using a Nikon epifluorescence microscope (filters B-3^{A}; excitation: λ = 420-490 nM; barrier: λ = 520 nM). One-hundred nuclei per sample were analyzed (treated and controls) and classified according to migration of fragments in classes 0, 1, 2 and 3 (Kobayashi, 1995): Class 0, cells without tail (not damaged) don´t damaged that don´t present tail; class 1, cells with tail smaller than the nucleus´ diameter; Class2, cells with size of tail among 1-2 times the nucleus´ diameter; class 3, cells with tail bigger than 2 times the nucleus´diameter.

Statistical analysis

Data were analyzed statistically by the χ^2 test (Pereira, 1991) for comparison of the total number of comets of each treatment with the values observed in the negative and positive controls.

Results and discussion

All tests conducted on water samples from the three collect stations indicated the presence of more alterations than observed in the negative control tests, with these waters being considered more genotoxic than the negative control. The genotoxicity data obtained by the comet assay for cells exposed to the water samples collected from the various stations showed lower values than those observed in the positive control (Table 1, Figure 2 and 3) and higher than those observed in the negative control. However, all of our results presented statistically significant values (0.01) regarding the genotoxic potential of the waters from the various collect sites. The comet assay on CHO-K1 cells appeared to be efficient in detecting the genotoxicity of waters contaminated with tannery effluents. Our data support those reported by Darroudi and Natarajan (1993) and McNamee et al.(2000), who stated that the comet assay is an efficient method for environmental biomonitoring.

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The damage indices (cells with comets) observed for stations FB and FC were usually higher than those observed at FA, with FB (the site of effluent dumping) showing the highest impairment (Table 1 and1Figure 2).

The FA site, corresponding to an upstream point in the river in relation to the dumping site and consequently a site not yet affected by the tannery effluent, did not show high indices of chromium contamination. Indeed the lowest values of total dissolved chromium (<0.01 to 0.05) were observed at this station (Table 2). Thus, the low indices of altered cells treated with the water from this site were correlated with the low chromium concentration existing there. The State Legislation of São Paulo-Brazil (CETESB,1995) establishes a maximum chromium level acceptable for class II and III waters (waters that receive effluents treated with substances considered not to be contaminants) of 0.05 mg/L. Our results agree with those reported by Pandrangi et al. (1995), who stated that the comet assay is an efficient method for the assessment of DNA damage induced by heavy metals.

According to Levis et al. (1978), chromium has a carcinogenic potential for man and animals by inducing cell transformations. Our results, suggest that the presence of higher chromium levels in the water can induce an increased percentage of damage to the DNA molecule (Figure 3).

At the site located upstream from the dumping site (FA) a greater genotoxic effect was observed for the winter, fall and summer. According to

the analyses performed on the samples from stations FB and FC (dumping site and downstream site, respectively), the same genotoxic effect of the waters was observed for all seasons of the year.

The present results indicate a greater water quality impairment by contaminants of tannery effluents, probably chromium, for the fall season, when the lowest rainfall rates were also observed (Figure 4). Thus, a prolonged draught may compromise even more the water resources that receive effluents with a genotoxic potential.

The chemical analyses of water samples obtained at the three collect stations during the four seasons of the year showed that the FB site (dumping site) in general presented the highest rates of dissolved ions, as can be seen in Table 2.

Conclusion

The present data suggest that the comet test has good sensitivity for the environmental monitoring of waters, thus being efficient for the assessment of the quality of waters contaminated with effluents containing residues of heavy metals such as chromium.

On the basis of the chromium concentrations present in the waters sampled at the various collect stations (Table 2), we can see that the genotoxic effects of chromium detected by the comet assay were already observed at concentrations as low as 0.01 mg/L, contradicting the toxicity

standards for chromium internationally established at levels starting from 0.05 mg/L.

Table 1 - Analysis of the changes observed in CHO-K1 cells treated with waters collected at distinct points along the stream which receives tannery effluents in the region of Franca/SP.

	Cells		Class					
	Normal	Altereted	0	1	2	3	Total of analysed cells	Score
Summer								
NC	296	4	296	4	0	0	300	4
PC	128	172	128	94	53	25	300	275
FA	272	28	272	24	4	0	300	32
FB	255	45	255	43	2	0	300	47
FC	222	78	222	49	27	2	300	109
Autumn								
NC	292	8	292	7	1	0	300	9
PC	240	60	240	45	11	4	300	79
FA	271	29	271	26	3	0	300	32
FB	246	54	246	48	4	2	300	62
FC	253	47	253	44	3	0	300	50
Winter								
NC	296	4	296	4	0	0	300	4
PC	204	96	204	65	24	7	300	134
FA	269	31	269	28	3	0	300	34
FB	253	47	253	38	6	3	300	59
FC	271	29	271	25	4	0	300	33
Spring								
NC	298	2	298	1	1	0	300	3
PC	191	109	191	75	26	8	300	151
FA	283	17	283	17	0	0	300	17
FB	276	24	276	21	3	0	300	27
FC	278	22	278	17	3	0	300	23

NC – Negative control; **PC –** Positive control; **FA –** Upstream from the site of effluent discharge; **FB –** Site of effluent discharge, and **FC –** Downstream from the site of effluent discharge.

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Table 2 - Determination of elements (ppm) by ICP-AES in water samples collected at three different points in the four seasons of the year.

		Chemical elements														
		Si	Ca	Sr	Mg	Pb	Fe	Mn	Cr	P	Al	Zn	Cu	Ba	Co	Ni	Cd
Summer																	
	FA	5.2	50.0	0.25	16.8	<0.05	0.21	0.07	0.05	1.5	0.33	0.11	0.01	0.04	<0.01	<0.01	<0.005
	FB	6.0	20.7	0.12	5.5	<0.025	0.8	0.10	0.38	2.20	0.43	0.04	0.01	0.05	<0.01	<0.01	<0.005
	FC	6.2	15.3	0.08	3.3	<0.025	0.3	0.04	0.23	0.11	0.14	0.05	<0.01	0.03	<0.01	<0.01	<0.005
Autumn																	
	FA	6.3	10.0	0.05	1.9	<0.025	0.3	0.01	<0.01	1.5	0.33	0.11	0.01	0.04	<0.01	<0.01	<0.005
	FB	5.6	18.8	0.10	4.6	<0.025	0.5	0.04	0.11	1,2	0.12	0.05	<0.01	0.04	<0.01	<0.01	<0.005
	FC	6.2	15.3	0.08	3.3	<0.025	0.3	0.04	0.05	1.1	0.07	0.01	<0.01	0.04	<0.01	<0.01	<0.005
Winter																	
	FA	6.4	9.2	0.05	2.1	<0.025	0.04	<0.01	<0.01	1.6	<0.05	0.01	<0.01	0.04	<0.01	0.01	<0.005
	FB	7.1	24.3	0.10	5.6	<0.025	0.18	0.05	0.02	1.82	0.05	0.02	<0.01	0.04	<0.01	0.01	<0.005
	FC	6.4	21.0	0.10	5.7	<0.025	0.15	0.08	0.02	1.22	<0.05	0.01	<0.01	0.05	<0.01	<0.01	<0.005
Spring																	
	FA	6.9	13.6	0.07	2.2	<0.025	0.16	0.03	<0.01	<0.1	0.10	0.01	<0.01	0.04	<0.01	<0.01	<0.005
	FB	7.3	36.3	0.15	9.1	<0.025	0.28	0.05	0.07	0.40	0.06	0.02	<0.01	0.04	<0.01	0.01	<0.005
	FC	7.2	25.5	0.12	6.3	<0.025	0.19	0.04	0.03	0.22	0.22	0.01	<0.01	0.06	<0.01	0.01	<0.005

FA – Upstream from the site of effluent discharge; **FB –** Site of effluent discharge, and **FC –** Downstream from the site of effluent discharge.

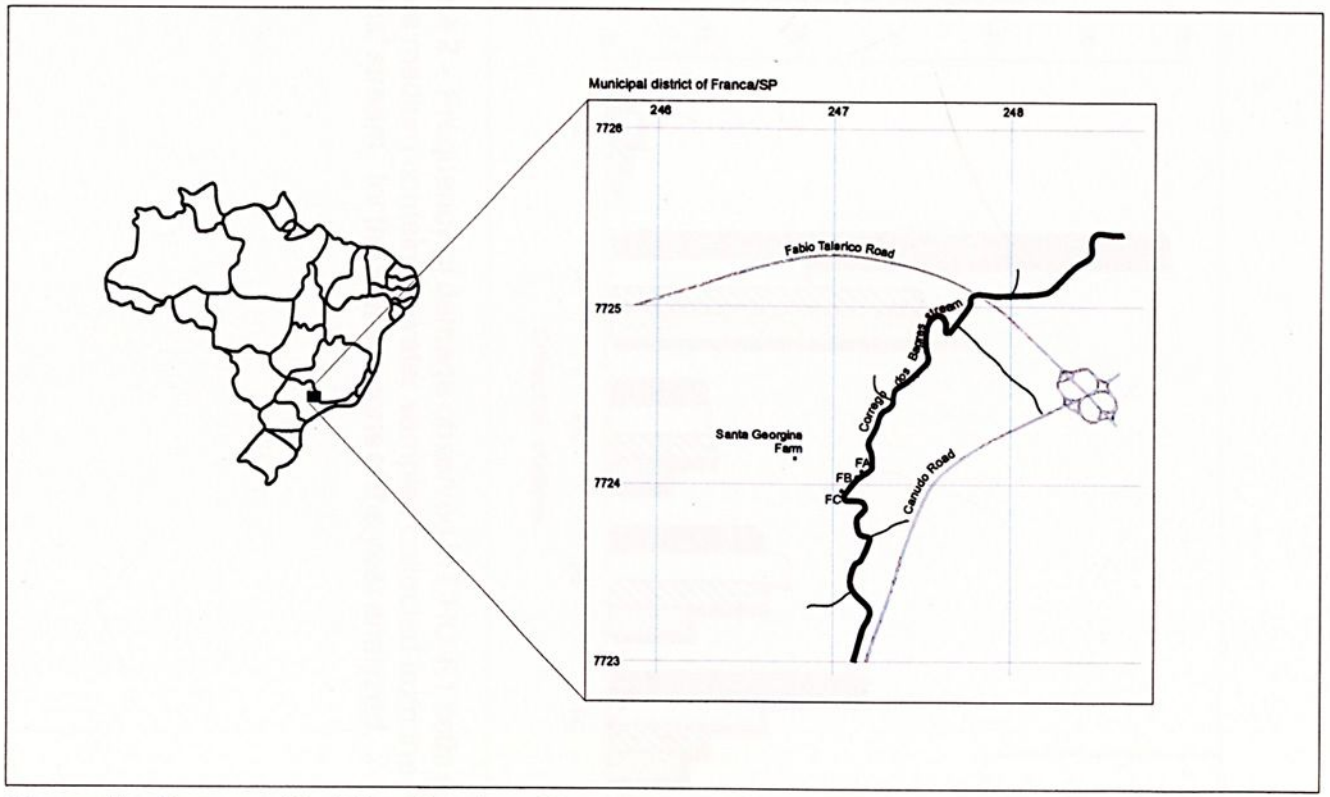

Figure 1 - Geographical localization of the collect site along the "Corrego dos Bagres" stream.

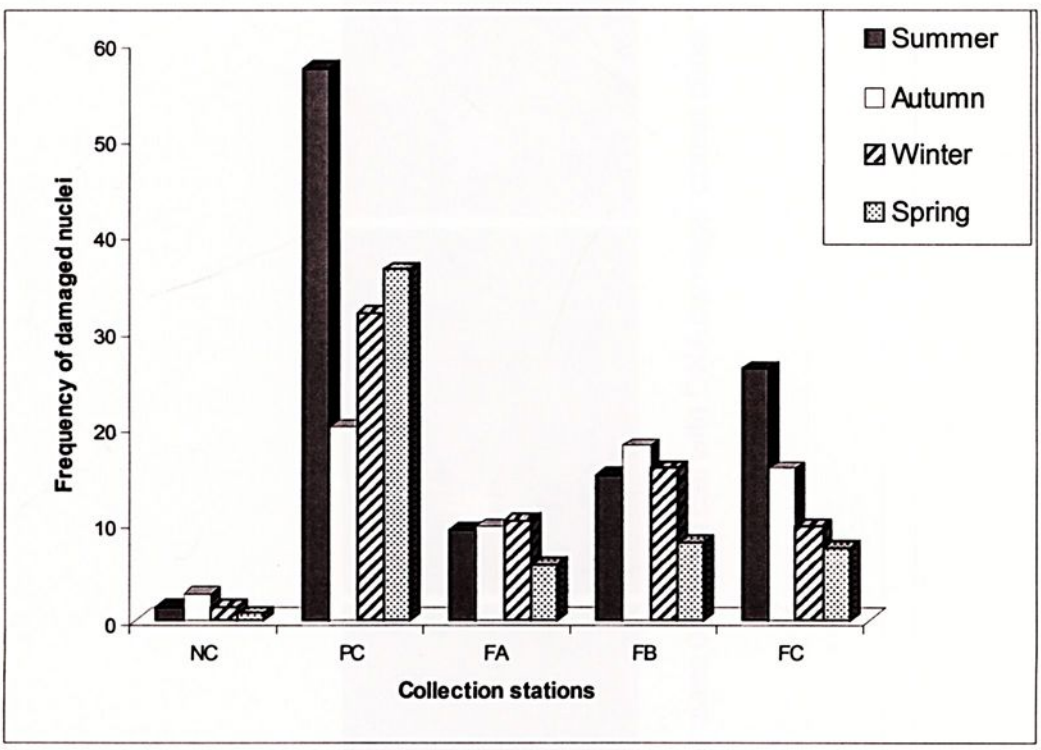

Figure 2 - Frequency of damage observed in CHO-K1 cells submitted to a culture medium containing water samples collected from the "Córrego dos Bagres" stream, for the four seasons of the year analyzed.

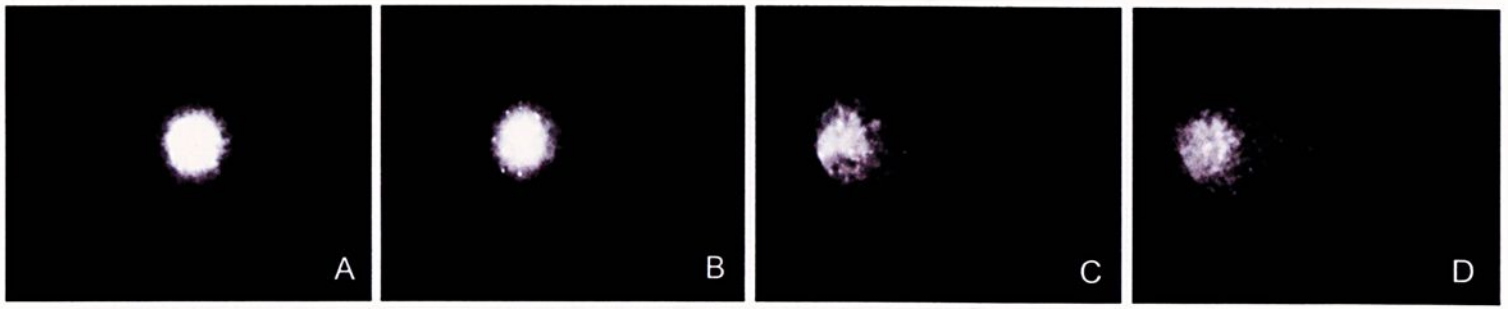

Figure 3 - Cell without DNA damage comet class 0 (A) and cell with DNA damage: comet class 1 (B), comet class 2 (C), comet class 3 (D).

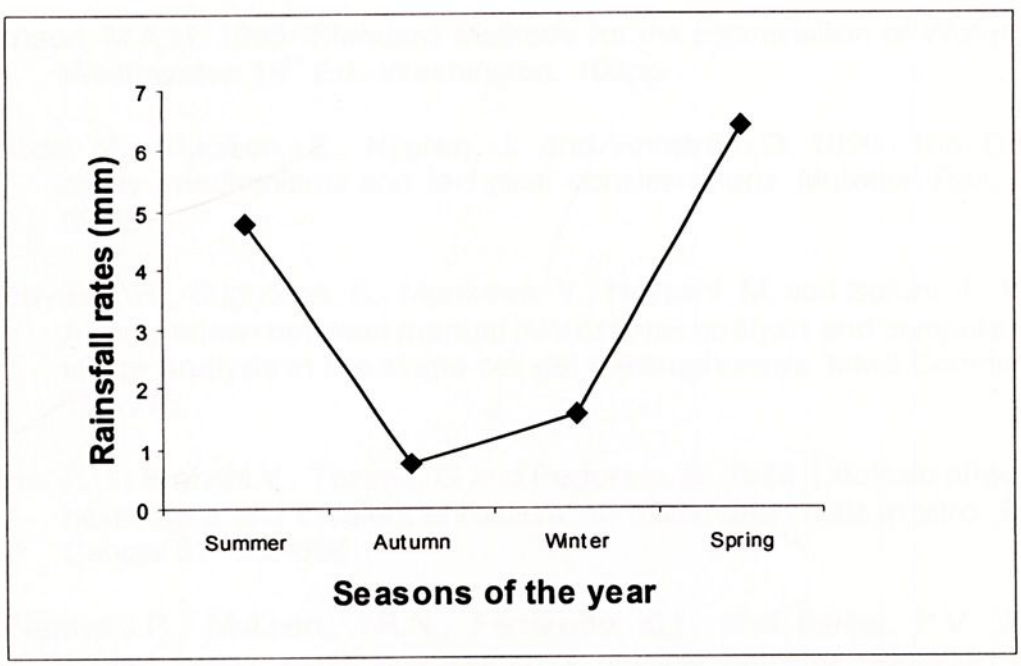

Figure 4 - Mean rainfall indices for the seasons of the year when water samples were collected from the "Córrego dos Bagres" stream.

References

CETESB - Compahia de Tecnologia de Saneamento Ambiental. 1995. Controle de Poluição Ambiental. Legislação Estadual. São Paulo-Brasil. 98pp.

Darroudi, F. and Natarajan, A.T. 1993. Metabolic activation of chemicals to mutagenic carcinogens by human hepatoma microsomal extracts in Chinese hamster ovary cells (*in vitro*). Mutagenesis **8**: 11-15.

Franson, M.A.H. 1995. Standard Methods for the Examination of Water and Wastewater. 19th Ed.. Washington. 103pp.

Klaude, M., Eriksson, S., Nygren, J. and Ahnström,G. 1996. The Comet assay: mechanisms and technical considerations. Mutation Res. **363**: 89-96.

Kobayashi, H., Sugiyama, C., Morikawa, Y., Hayashi, M. and Sofuni, T. 1995. A comparison between manual microscopic analysis and computerized image analysis in the single cell gel electrophoresis. MMS Commun **3**: 103-115.

Levis, A.G., Blanchi,V., Tamino, G and Pegoraro, B. 1978. Citotoxic effects of hexavalent and trivalent chromium on mammalian cells *in vitro*. Br. J. Cancer **37**: 389-396.

McNamee,J.P., McLean, J.R.N., Ferrarotto, C.L. and Bellier, P.V. 2000. Comet assay: rapid processing of multiple samples. Mutation Res. **466**: 63-69.

Mitchelmore, C.L. and Chipman, J.K. 1998. DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. Mutation Res. **399**: 135-147.

Nacci,D., Nelson, S., Nelson, W. and Jackim, E. 1992. Application of the DNA alkaline unwinding assay to detect DNA strand breaks in marine bivalves. Mar. Environ. Res. **33**: 83-100.

Olive, P.L. and Banáth, J.P. 1993. Induction and rejoining of radiation-induced DNA single-strand breaks: 'tail moment' as a function of position in the cell cycle. Mustation Res. **294**: 275-283.

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Pandrangi, R., Petras, M., Ralph, S. and Vrzoc, M. 1995. Alkaline single cell gel (Comet) assay and genotoxicity monitoring using bullheads and carp. Environ. Mol. Mutag. **26**: 345-356.

Pereira, A. A. B. 1991. Testes estatísticos para comparar proporções em problemas de citogenética. *In*: Rabello-Gay, M.N., Rogrigues, M. A. L. R e Monteleone-Neto, R. (eds). Mutagênese, Carcinogênese e Teratogênese: Métodos e Critérios de Avaliação, Ver. Brasil. De Genética 113-121.

Tice, R. R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J. C. and Sasaki, Y.F. 2000. Single Cell Gel/Comet Assay: Guidelines for *in vitro* and *in vivo* Genetic Toxicology Testing. Environ. Mol. Mutag. **35**: 206-221.

White, P.A. and Rasmussen. 1998. The genotoxic hazards of domestic wastes in surface waters. Mutation Res. **410:** 223-236.

8. – ARTIGO 06

EVALUATION OF THE GENOTOXIC POTENTIAL DUE TO THE ACTION OF AN EFFLUENT CONTAMINATED WITH CHROMIUM, BY THE COMET ASSAY IN CHO-K1 CULTURES

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Abstract

The comet assay technique has been considered to be more efficient in the biomonitoring of aquatic environments that the micronucleus and sister chromatid exchange techniques. The comet assay has been used to determine breaks in the DNA strands of organisms exposed to pollutants with a genotoxic potential. The comet technique was applied to CHO-K1 cells in order to evaluate the genotoxic potential of the waters of the Sapucaizinho River (Municipality of Patrocínio Paulista, State of São Paulo, Brazil), which receive tannery effluents and therefore are contaminated with chromium. The results indicated high genotoxicity of the waters collected at sites located downstream from the emission of tannery effluents, where the concentration of chromium was found to be high.

Key words: Comet assay; Chromium; Genotoxicity

INTRODUCTION

Many potentially toxic chemical substances, both of natural origin or due to human actions, are released into the environment. Due to the increasing environmental exposure to these agents, there has been increasing interest in biomonitoring terrestrial and aquatic ecosystems, especially in regions compromised by chemical pollution (Silva et al., 2003).

Environmental pollution due to heavy metals represents a serious problem because of its high toxicity and of the bioaccumulation ability of these agents (Guecheva et al., 2001; Pruski and Dixon, 2002). Lee and Steinert (2003) and Matsumoto (*in press)* reported that chromium represents a dangerous heavy metal which can directly damage DNA by promoting strand breaks or DNA-protein crosslinks, or by acting on the generation of reactive oxygen species.

The comet assay or single cell gel electrophoresis (SCGE) is a simple, sensitive and rapid technique applied to isolated cells for the detection of DNA damage (Fairbain et al., 1995; Klaude et. al., 1996; Speit and Hartmann, 1999), and represents a highly efficient method for application to toxicologic genetics, especially ecogenotoxicology (Pavlica et al., 2001). This technique has been applied by Mitchelmore and Chipman (1998) and Kassie et al. (2000) to studies of DNA repair, of the effect of biological radiation, and of apoptosis.

The comet assay technique has been considered to be more efficient than the micronucleus or sister chromatid exchange technique as a bioindicator of alterations of aquatic environments. Lee and Steinert (2003) have been using this technique in several *in vivo* and *in vitro* studies for the determination of strand breaks in the DNA of aquatic organisms exposed to pollutants with a genotoxic potential.

The same efficiency of this technique was reported by Padangri et al. (1995) when assessing sediments contaminated with heavy metals in the Great Lakes (Canada), where they observed induction of DNA damage caused by these contaminants. Nacci et al. (1992) also reported a reduction in DNA strand breaks in organisms transferred from sites with sediments contaminated with heavy metals to control sites free from these pollutants.

Using the comet assay in human lymphocytes, Rajaguru et al. (2002) investigated the genotoxic potential of waters polluted with textile industry effluents in India.

Biomonitoring studies were conducted by Avishai et al. (2002) on the Kishon River (Israel) using the comet assay applied to a culture of the fish hepatoma cell line RTH-149. The authors reported a high genotoxicity of the water samples analyzed, showing the efficiency of this technique for the assessment of the genotoxic potential of an aquatic environment.

The efficiency of the comet assay was also demonstrated for CHO-K1 cell cultures (Matsumoto et al., *in press*) exposed to water from the Córrego

dos Bagres Stream (Franca/SP-Brazil) that receives tannery effluents. The authors observed that chromium had a genotoxic effect at concentrations of 0.01 mg/L, below the limit permitted by International Legislation (0.05 mg/L), demonstrating that this heavy metal has a high polluting action on water resources and a high genotoxic potential.

The municipality of Patrocínio Paulista-SP is a region with intense industrial activity related to leather processing (tanning). Several toxic substances, chromium among them, are used for leather tanning and are considered to be harmful to the environment. In the present study, using the comet assay technique applied to CHO-K1, we assessed the genotoxic potential of waters from the Sapucaizinho River, municipality of Patrocínio Paulista – State of São Paulo – Brazil, which receive tannery effluents and are therefore contaminated with chromium.

MATERIALS AND METHODS

Sample collect

Water collects were performed during the four seasons of the year 2002 at three different stations along the Sapucaizinho river, Municipal district of Patrocínio Paulista/SP. The collect sites selected were: before the site of tannery effluent dumping (1000 m upstream) – PA, dumping site – PB, and after the dumping site (1000 m downstream) – PC (Figure 1). At each collect, 2 liters of water were obtained at a depth of 30 cm according to routine

criteria for chemical analyses. The water samples were placed in plastic bottles and carried in an insulated box with ice to Universidade Estadual Paulista – UNESP – Rio Claro /SP.

Physicochemical analysis of the water

The analyses were based on the Standard Methods for the Examination of Water and Wastewater (Franson, 1995).

For cation determination, the samples were first acidified with HNO_3, pH 1, and the cations were analysed sequencialy by inductively coupled plasma atomic emission spectrometry (ICP-AES) with ultrasonic nebulization. The following elements were determined: calcium, magnesium, strontium, silicon, iron, manganese, aluminium, zinc, chromium, cobalt, nickel, lead, cadmium, phosphorus, copper, and barium.

The standard solutions used to construct the calibration curves for the elements analyzed in 0.1% HNO_3 medium were obtained by appropriate dilutions of the standard solutions of 1000 ppm Titrisol (Merck).

Culture conditions and treatments

CHO-K1 cells were cultured in D-Mem + HAMF10 (1:1) medium supplemented with 10% Bovine Serum Albumin at 37ºC. For the treatments a concentrated culture medium (stock) was prepared, to be diluted 3:1 with the

water samples collected. The water samples collected at each station were added to the concentrated culture medium at the treatment time.

After 24 hours of culture in normal medium, CHO-K1 cells were kept for 2 hours (37ºC) in the mixture of medium and water collected from the stream, whose pH was adjusted to 7.2 before treatment. The positive control was treated with methylmethanesulfonate (MMS) at the concentration of 10^{-4}M and the negative control was only kept in culture medium. Three independent experiments were performed.

Comet assay

The slides for microscope observation were mounted with 20 μl of the above cellular suspension and 120 μl of low melting point agarose at 37ºC. The slides were bathed in a lysis solution (1 ml of Triton X-100, 10 ml of DMSO and 89 ml of lysis stock solution [2.5 M NaCl, 100 mM EDTA, 10mM Tris, pH 10, ~8 g solid NaOH, and 10 g lauryl sarcosinate, pH 10]), in the dark, in a refrigerator at about 8ºC for 1 hour.

After lysis, the slides were placed in buffer containing 0.3 N NaOH and 1 mM EDTA, pH>13, for 20 minutes for DNA unwinding. Electrophoresis was performed for 20 minutes at 25 V, 300 mA (~1.0 V/cm) and the slides were them neutralized for 15 minutes in 0.4 M Tris, fixed in absolute ethanol for 10 minutes, and stained with ethidium bromide (0.02 μg/ml).

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Comet assay analysis

The comet observations were performed at 400X using a Nikon epifluorescence microscope (filters B-3^{A}; excitation: λ = 420-490 nM; barrier: λ = 520 nM). One-hundred nuclei per sample were analyzed (treated and controls) and classified according to migration of fragments in classes 0, 1, 2 and 3 (Kobayashi, 1995): Class 0, cells without tail (not damaged) don't damaged that don't present tail; class 1, cells with tail smaller than the nucleus' diameter; Class2, cells with size of tail among 1-2 times the nucleus' diameter; class 3, cells with tail bigger than 2 times the nucleus'diameter.

Statistical analysis

Data were analyzed statistically by the χ^2 test (Pereira, 1991) for comparison of the total number of comets of each treatment with the values observed in the negative and positive controls.

RESULTS AND DISCUSSION

CHO-K1 cultures treated with water samples collected from predetermined sites along the Sapucaizinho River and submitted to the comet assay presented DNA damage during all seasons of the year.

The frequency of damage observed for all the collection sites during all seasons was lower than that observed in the positive control and higher than

that observed in the negative control (except for the summer). These data agree with those described by Avishai et al. (2002) and Matsumoto et al. (*in press)*, who reported that the comet assay technique applied to cell cultures is efficient for application to the biomonitoring of water resources.

During the summer, the genotoxic effect observed at the three collection sites was not significant compared to control (Table 1). These results may be related to the high rainfall observed during this period of collection (Figure 2). During the other seasons, damage to the cell nuclei was observed, with the highest frequency always being recorded for the PB site. This high frequency of DNA damage at the PB site can be explained by the fact that this site corresponded to the exact place where the tannery effluent was discharged. Coincidentally, it was also this site that presented the highest concentration of total chromium, as shown in Table 2.

The highest harmful effect on the Sapucaizinho River was observed during the winter, when the rainfall rate was the lowest among all seasons of the year investigated (Table 1 and Figure 2). In winter, the highest genotoxic effect was observed for the PB site (Table 1 and Figures 3 and 4). No class 3 nuclear damage was observed in any of the samples analyzed.

The PA site (upstream to the effluent) presented a lower frequency of nuclear damage compared to the PB site. This can be explained by the location of the PA site itself, since at this site there was no emission of

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tannery effluents and therefore no influence of the chromium residue was present (Table 1, Figure 3).

Chromium is considered to be a carcinogen because it induces lesions in the DNA molecule (Debetto et al., 1982; Matsumoto, 2003). The present data indicate a directly proportional relationship between the frequency of DNA damage and the concentration of chromium residues, thus confirming that chromium is a heavy metal with the potential to induce cell transformations, as also reported by Von Burg and Liu (1993) and in a previous paper by our group (Matsumoto, 2003).

According to the Legislation of the State of São Paulo (1995) - Brazil, the chromium residue concentration permitted in industrial effluents discharged into Class II and III rivers is up to 0.05 mg/L. The present data indicate the presence of a genotoxic effect on CHO-K1 cell cultures as determined by the comet assay in water samples with chromium residue concentrations of less than 0.005 mg/L, confirming results obtained in a previous study (Matsumoto et al., *in* press). These reports indicate that the genotoxic effects of chromium are already perceptible at concentrations of less than 0.05 mg/L (Table 2). Thus, leather tanneries that discharge effluents within the limits of current Brazilian Legislation may compromise the water quality of the rivers into which they dump such effluents, consequently endangering the organisms, and even man, who use these water for drinking purposes. All of our results indicate a genotoxic potential of the waters

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collected from the Sapucaizinho River after they receive the tannery effluent during different seasons of the year.

The comet test proved to be efficient for the monitoring of waters that receive tannery effluents and that are contaminated with chromium. The same efficiency of the technique for environmental monitoring was also demonstrated by Rajaguru et al. (2002) when they investigated the potential for genotoxicity induction of textile industry effluents, by Nacci et al. (1992) and Padangri et al. (1995) who assessed the contamination of sediments with heavy metals, and by Matsumoto et al. (*in press*) who investigated the genotoxicity of the heavy metal chromium present in tannery effluents.

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Table 1 - Analysis of the changes observed in CHO-K1 cells treated with waters collected at distinct points along the river which receives tannery effluents in the region of Patrocínio Paulista/SP.

		Class				Cells		
		0	1	2	3	analysed	altereted	Escore
Summer	NC	95.00±7.81	4.00±6.08	1.00±1.73	0.00±0.00	300	5.00	6
	PC	87.33±2.08	8.67±2.08	2.67±0.58	1.00±0.00	300	12.33	17.01
	PA	98.33±0.58	1.67±0.58	0.00±0.00	0.00±0.00	300	1.66	1.67
	PB	97.00±2.00	2.00±1.73	1.00±1.00	0.00±0.00	300	3.00	4.00
	PC	97.33±1.15	2.33±0.58	0.50±0.71	0.00±0.00	300	2.66	3.33
Autumn	NC	100±0.00	0.00±0.00	0.00±0.00	0.00±0.00	300	0.00	0.00
	PC	93.00±2.65	6.00±2.65	0.67±0.58	0.33±0.58	300	7.00	8.33
	PA	96.67±1.15	3.33±1.15	0.00±0.00	0.00±0.00	300	3.33	3.33
	PB	95.00±1.73	4.00±0.00	1.00±1.73	0.00±0.00	300	5.00	6.00
	PC	97.00±1.00	2.67±0.58	0.33±0.58	0.00±0.00	300	3.00	3.66
Winter	NC	99.33±1.15	0.67±1.15	0.00±0.00	0.00±0.00	300	0.66	0.67
	PC	83.33±4.04	14.33±2.52	3.67±1.15	2.00±1.00	300	19.33	27.67
	PA	94.00±3.00	5.00±1.73	1.00±1.73	0.00±0.00	300	6.00	7.00
	PB	91.00±1.00	7.33±0.58	1.67±1.53	0.00±0.00	300	9.00	10.67
	PC	96.67±2.31	3.00±1.73	0.33±0.58	0.00±0.00	300	3.33	3.99
Spring	NC	100±0.00	0.00±0.00	0.00±0.00	0.00±0.00	300	0.00	0.00
	PC	84.67±0.58	13.00±0.00	2.00±1.00	0.33±0.58	300	15.33	17.99
	PA	96.00±1.73	3.33±1.53	1.00±0.00	0.00±0.00	300	4.00	5.33
	PB	94.00±2.65	5.00±1.00	1.00±1.73	0.00±0.00	300	6.00	7.00
	PC	98.33±1.53	1.67±1.53	0.00±0.00	0.00±0.00	300	1.66	1.67

NC- negative control; **PC**- positive control; **PA**- uspstream from the site of effluent discharge; **FB**- site of effluent discharge; **FC**- downstream from the site of effluent discharge.

Table 2 - Determination of elements (ppm) by ICP-AES in water samples collected at three different points in the four seasons of the year.

		Chemical elements										
		Pb	Fe	Cd	Cr	P	Al	Zn	Cu	Ba	Co	Ni
Summer												
	PA	<0.025	1.1	<0.005	<0.01	<0.1	0.12	<0.01	<0.01	0.02	<0.01	<0.01
	PB	<0.025	1.2	<0.005	0.01	<0.1	0.2	<0.01	<0.01	0.03	<0.01	<0.01
	PC	<0.025	1.1	<0.005	<0.01	<0.1	0.1	<0.01	<0.01	0.02	<0.01	<0.01
Autumn												
	PA	<0.012	0.96	<0.003	<0.005	<0.1	0.05	<0.005	<0.005	0.02	<0.005	<0.005
	PB	<0.012	0.81	<0.003	0.005	<0.1	0.1	<0.005	<0.005	0.03	<0.005	<0.005
	PC	<0.012	0.85	<0.003	<0.005	<0.1	0.07	<0.005	<0.005	0.02	<0.005	<0.005
Winter												
	PA	<0.02	0.57	<0.003	<0.005	<0.1	0.1	<0.005	<0.005	0.02	<0.005	<0.005
	PB	<0.02	0.13	<0.003	0.17	0.48	0.35	0.01	<0.005	0.01	<0.005	<0.005
	PC	<0.02	0.43	<0.003	<0.005	<0.1	0.08	<0.005	<0.005	0.02	<0.005	<0.005
Spring												
	PA	<0.02	2.6	<0.003	<0.005	<0.1	4.17	0.01	0.02	0.02	<0.005	<0.005
	PB	<0.02	3.15	<0.003	<0.005	0.11	5.27	<0.005	0.02	0.02	<0.005	<0.005
	PC	<0.02	3.24	<0.003	<0.005	0.11	5.23	<0.005	0.01	0.01	<0.005	<0.005

PA- upstream of the river; **PB**- disposal site; **PC**- downstream of the river

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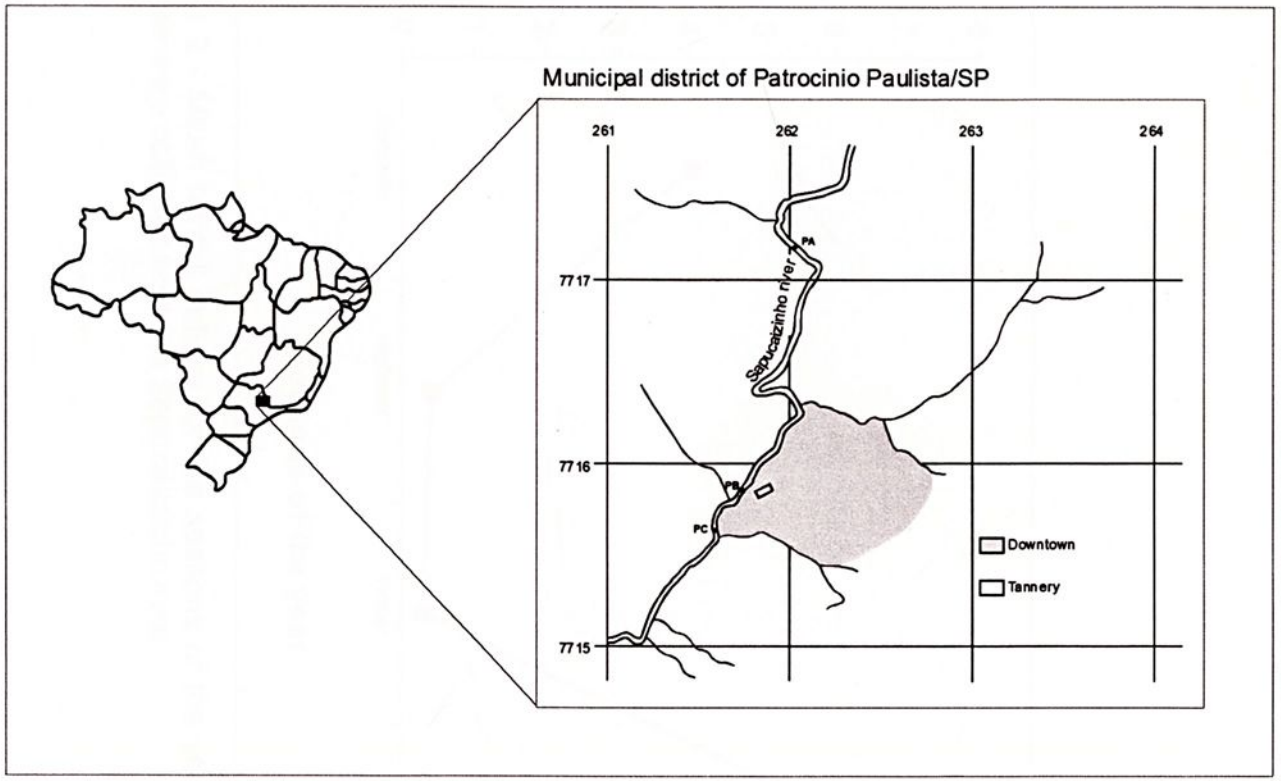

Figure 1 - Geographical localization of the collected site along the Sapucaizinho river.

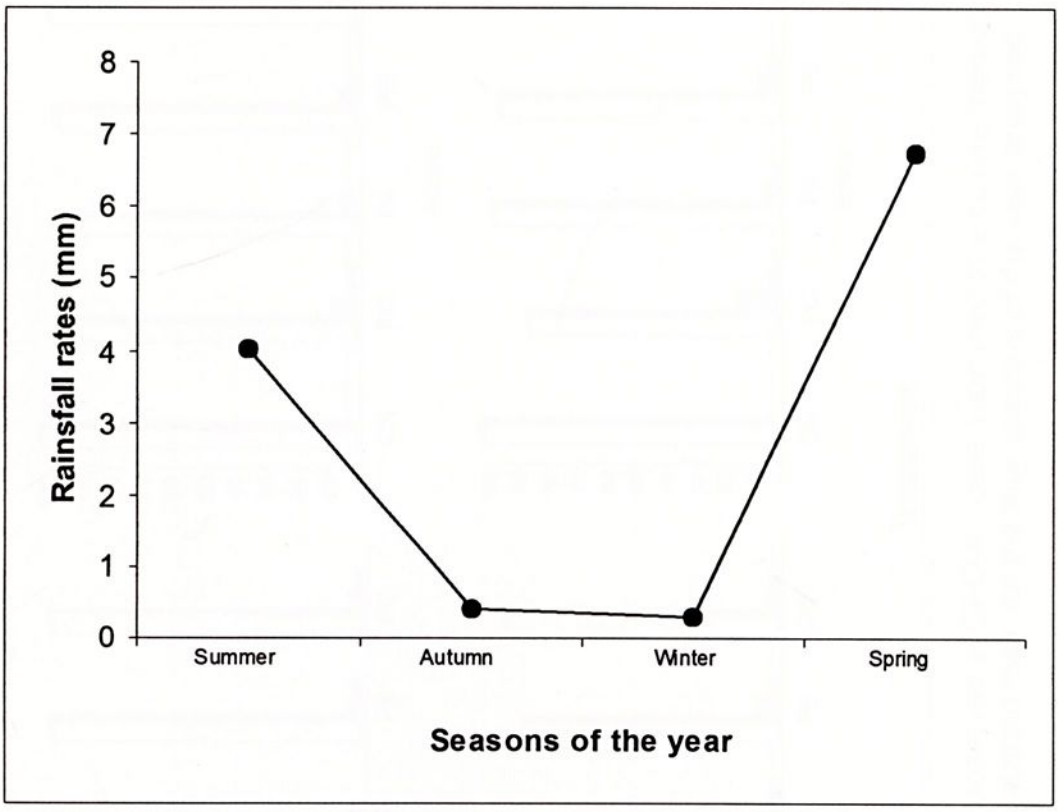

Figure 2 - Mean rainfall indices for the seasons of the year when water samples were collected from the Sapucalizinho river.

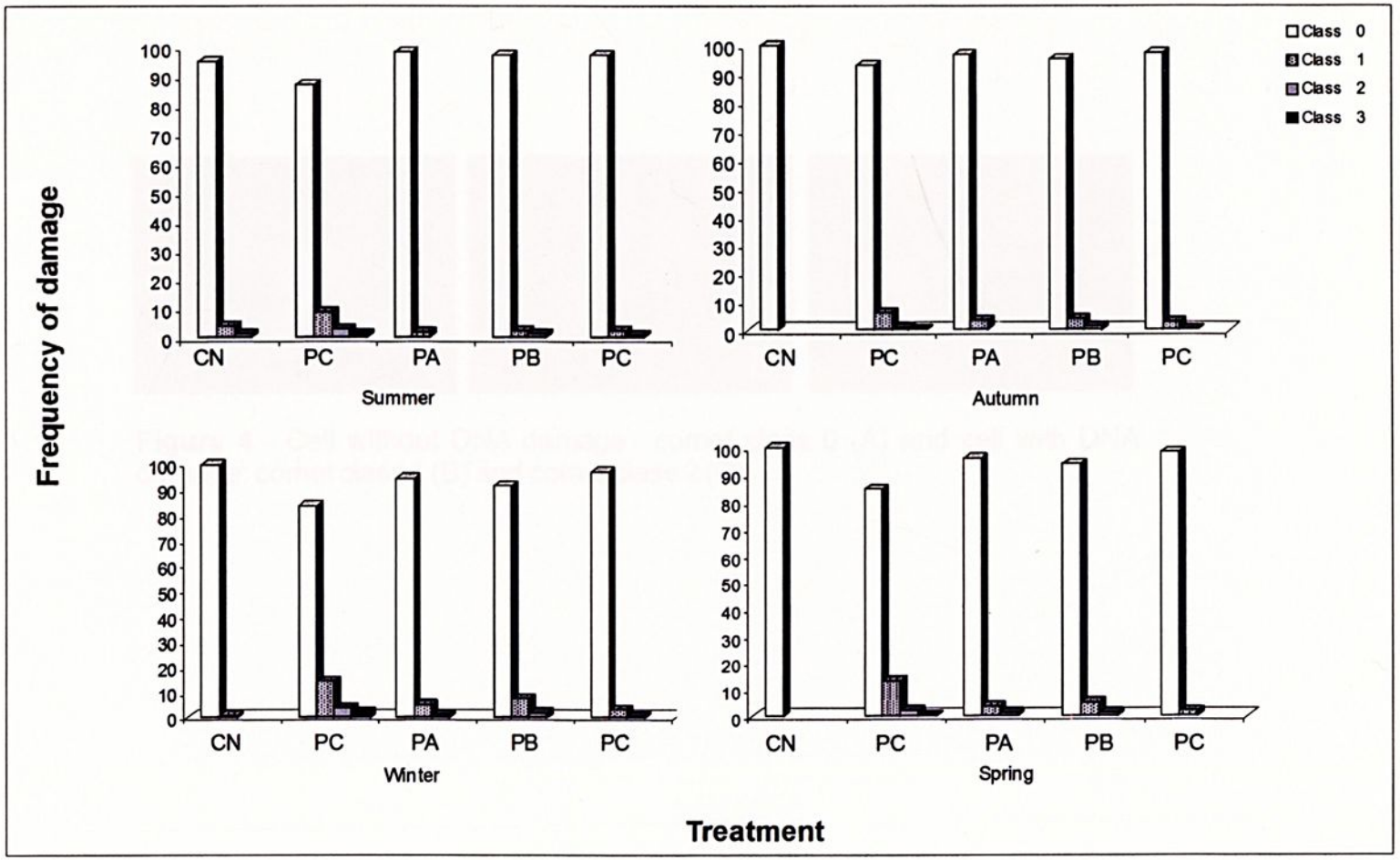

Figure 3 - Frequency of damage observed in CHO-K1 cells submitted to a culture medium containing water samples collected from the Sapucaizinho river, for the four seasons of the year analysed.

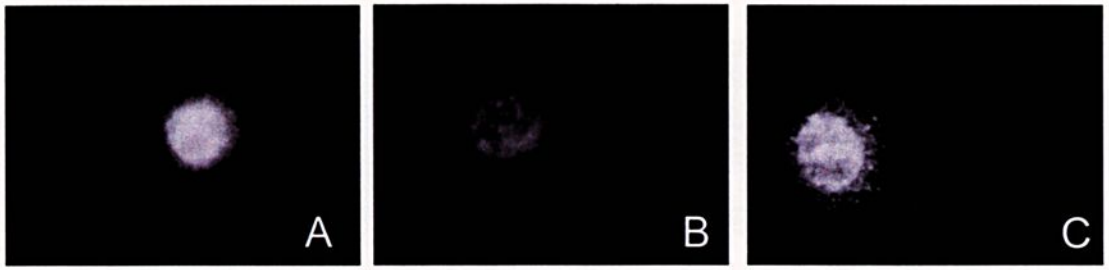

Figure 4 - Cell without DNA damage comet class 0 (A) and cell with DNA damage: comet class 1 (B) and comet class 2 (C).

REFERENCES

Avishai, N.; Rabinowitz, C.; Moiseeva, E.; Rinkevich, B., 2002- *Genotoxicity of the Kishon river, Israel: the apllication of an in vitro cellular assay*. Mutation research, 518: 21-37.

Debetto, P.; Toso, R.D.; Varotto, R.; Bianchi, V.; Luciani, S., 1982 - *Effects of potassium dichromate on atp content of mammalian-cells cultured in vitro*. Chemico-biological interactions, 41: 15-24.

Farbain, D. W.; Olive, P. L.; O'Neil, K. L., 1995 - *The comet assay: a comprehensive review*. Mutation Research, 339: 37-59.

Franson, M. A. H., 1995 - Standard Methods for the Examination of Water and Wastewater. 19th Ed.. Washington.

Guecheva, T.; Henriques, J. A. P; Erdtmann, B., 2001 - *Genotoxic effects of copper sulphate in freshqater planarian in vivo, studied with single-cell gel test (comet assay)*. Mutation Research, 497: 19-27.

Kassie, F.; Parzefall, W.; Knasmüller, S., 2000 - *Single cell gel electrophoresis assay: a new techinique for human biomonitoring studies*. Mutation research, 463: 13-31.

Klaude, M.; Eriksson, S.; Nygren, J.; Ahnström, G. 1996 - *The comet assay: mechanisms and technical considerations*. Mutation research, 363: 89-96.

Kobayashi, H., Sugiyama, C., Morikawa, Y., Hayashi, M.; Sofuni, T.,1995 - *A comparison between manual microscopic analysis and computerized image analysis in the single cell gel electrophoresis*. MMS Commun, 3: 103-115.

Lee, R. F.; Steinert, S., 2003 - *Use of the single cell gel eletrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals*. Mutation Research, 544: 43-64.

Matsumoto, S.T. 2003. *Efeitos tóxicos e genotóxicos de metais pesados, especificamente do cromo trivalente e hexavalente*. Monografia (Doutorado) Universidade Estadual Paulista. São José do Rio Preto-SP.

Matsumoto, S. T.; Mantovani, M. S.; Mallaguti, M. I.; Marin-Morales, M.A., *in press - Investigation of the genotoxic potential of the waters of a river receiving tannery effluents by means of the in vitro comet assay*. Cytologia.

Mitchelmore, C. L.; Chipman, J. K., 1998 - *DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring*. Mutation Research, 399: 135-147.

Nacci, D.; Nelson, S.; Nelson, W.; Jackim, E., 1992 - *Application of the DNA alkaline unwinding assay to detect DNA strand breaks in marine bivalves*. Marine Environmental Research, 33: 83-100.

Pandrangi, R.; Petras, M.; Ralph, S.; Vrzoc, M. 1995 - *Alkaline single cell gel (Comet) assay and genotoxicity monitoring using bullheads and carp*. Environmental Molecular Mutagenic, 26: 345-356.

Pavlica, M.; Klobucar, G. I. V.; Mojas, N.; Erben, R.; Papes, D., 2001 - *Detection of DNA damage in haemocytes of zebra mussel using comet assay*. Mutation Research, 490: 209-214.

Pruski, A: M.; Dixon, D. R., 2002. *Effects of cadmium on nuclear integrity and DNA repair efficiency in the gill cells of Mytilus edulis L.* Aquatic Toxicology, 57: 127-137.

Pereira, A. A. B., 1991 - Testes estatísticos para comparar proporções em problemas de citogenética. In: Rabello-Gay, M.N., Rogrigues, M. A. L. R e Monteleone-Neto, R. (eds). Mutagênese, Carcinogênese e Teratogênese: Métodos e Critérios de Avaliação, Revista Brasileira de Genética, 113-121.

Rajaguru, P.; Vidya, L.; Baskarasethupathi, B.; Kumar, P.A.; Palanivel, M.; Kalaiselvi, K., 2002 - *Genotoxicity evaluation of polluted ground water in human peripheral blood lymphocytes using the comet assay.* Mutation research, 517:29-37.

Silva, J.; Heuser, V.; Andrade, V., 2003 - *Biomonitoramento Ambiental. In Silva, J.; ERDTMANN, B.; HENRIQUES, J. A. P.* Genética Toxicológica. Porto Alegre: Alcance, 8: 166-180.

Speit, G.; Hartmann, A., 1999 - *The comet assay (Single-cell gel test)- A sensitive genotoxicity test for the detection of DNA damage and repair. In HENERSON, D. S. (ed.)*. Methods in molecular biology. V.113, DNA Repair Protocols: Eukaryotic Sistems, Humana Press, Totowa, NJ, 203-212.

Von Burg, R.; Liu, D., 1993 - *Chromium and hexavalent chromium*. Journal of applied toxicology, 13: 225-230.

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9. – DISCUSSÃO GERAL

9.1 – Organismos teste

Foram realizadas análises de citotoxicidade e genotoxicidade utitlizando-se *Allium cepa*, *Oreochromis niloticus* e células de ovário de Hamster (CHO-K1), como organismos teste, em amostras de água coletadas no rio Sapucaizinho-Município de Patrocínio Paulista/SP e no Córrego dos Bagres-Município de Franca/SP, durante as estações do ano de 2001 e 2002.

O sistema de *A. cepa* foi utilizado para avaliar as variações, no ciclo celular, decorrentes da ação de agentes tóxicos presentes nas águas das diversas coletas realizadas em rios da região de Franca, em quatro diferentes tratamentos (contínuo, descontínuo agudo, descontínuo crônico e recuperação). Para a comparação dos resultados, foi realizado um controle negativo em água Milli-Q e um controle positivo em solução aquosa de cromo trivalente. As coletas foram feitas durante os períodos de seca (maio a outubro) e de chuva (novembro a abril) dos anos de 2001 e 2002.

Os bioensaios com *O. niloticus* foram desenvolvidos utilizando-se as amostras de águas do Córrego dos Bagres, coletadas na estação chuvosa do ano de 2001. As análises foram feitas pelo sistema de micronúcleo e pelo ensaio do cometa.

As células de ovário de Hamster (CHO-K1) foram submetidas, *in vitro*, às amostras de água coletadas no Córrego dos Bagres, no ano de 2001, e às amostras coletas em 2002, no rio Sapucaizinho, para que fossem avaliados os seus potenciais genotóxicos, pela técnica do ensaio do cometa. Para esse sistema, foi utilizado, como controle positivo, o metil metano sulfanato (MMS).

A escolha dos organismos teste seguiu indicação de outros estudos (GÓMEZ-ARROYO e VILLALOBOS-PIETRINI, 1983; AL-SABTI, 1986; HOSE et al., 1987; PRESTON et al., 1987; METCALFE, 1988; RABELO-GAY et al., 1991; LIU et al.,1992; AL-SABTI e METCALFE, 1995; MINISSI et al., 1997; SMAKA-KINCL et al., 1997; DASHWOOD e BARILEY, 1998; SAHI et al., 1998; GROVER e KAUR, 1999; MORAES e JORDÃO, 2001), que mostraram a efetividade desses organismos na avaliação de mutagênese ambiental.

9.2 - Análise química

Foram realizadas análises químicas em todas as amostras de água coletadas no rio Sapucaizinho – Município de Patrocínio Paulista/SP, e no Córrego dos Bagres – Município de Franca/SP, para determinar a concentração dos elementos dissolvidos nestes recursos hídricos em estudo.

O cromato é o químico utilizado pelos curtumes para tratar o couro de boi. Conseqüentemente, resíduos desse cromato são encontrados nos efluentes dos curtumes, lançados nos rios. Pelas análises químicas, o agente

contaminante, em maior concentração, presente nos recursos hídricos da região de Franca, foi o cromo. O Cr III é tido como um metal pesado, capaz de induzir uma variedade de danos à molécula de DNA (MATSUMOTO, 2003).

Os resultados revelaram que os pontos PB-L, PB-T e PC, do rio Sapucaizinho, e os pontos FB e FC, do Córrego dos Bagres, foram os que apresentaram maiores índices de contaminação pelo cromo. Esses altos índices podem ser explicados pela localização desses pontos, pois eles correspondem aos próprios locais de despejo dos efluentes dos curtumes, ou pontos à sua jusante. A alta concentração do cromo total nesses pontos pode evidenciar o comprometimento da qualidade das águas dos recursos hídricos analisados. Os pontos PA e FA, à montante do efluente, não apresentaram índices elevados de cromo total por se localizarem antes dos efluentes dos curtumes, não sofrendo influências dessa contaminação.

9.3 - Análise do efeito citotóxico (índice mitótico)

Foram realizadas análises dos índices mitóticos das células meristemáticas de *A. cepa,* tratadas com as amostras de águas coletadas nos rios em estudo, para se avaliar o possível efeito citotóxico dos resíduos de cromo presentes nesses ambientes aquáticos.

Quando os índices mitóticos apresentam valores superiores aos encontrados no controle negativo, significa que houve uma indução no

processo de divisão celular. Essa indução pode ocasionar uma proliferação descontrolada das células e resultar na formação de neoplasias. A redução dos índices mitóticos pode, muitas vezes, comprometer o desenvolvimento e crescimento dos organismos.

De acordo com Smaka-Kincl et al. (1997), a análise dos índices mitóticos constitui uma ferramenta confiável para estudos de monitoramento de águas superficiais poluídas. Os autores ainda afirmam que o nível de citotoxicidade de águas poluídas pode ser determinado pela taxa de redução ou de aumento desses índices mitóticos.

A análise dos índices mitóticos das células meristemáticas de *A. cepa*, submetidas às amostras de água coletadas em diferentes pontos do Córrego dos Bagres, revelou que houve uma redução dos índices para todas as amostras, tanto no período de chuva como no período de seca, nos anos de 2001 e 2002. Porém, para as amostras coletadas no rio Sapucaizinho, não houve registro de redução do índice mitótico para os pontos PB-T e PC do tratamento contínuo, PC do tratamento agudo, e PC do tratamento crônico, para as amostras coletadas no período chuvoso do ano de 2001. Para todas as amostras coletadas no ano de 2002, houve a redução do índice mitótico, com exceção do ponto PA do tratamento contínuo. Para as amostras de água coletadas no período de seca neste rio, não foi registrada a redução do índice mitótico para o ponto PA do tratamento contínuo (2001).

Nossos resultados sugerem uma tendência de indução de efeito citotóxico, para as águas do rio Sapucaizinho, coletadas no período de seca. Todos os tratamentos realizados com todos os pontos de coleta deste período apresentaram uma redução do índice mitótico, com exceção do ponto à montante do despejo do efluente (PB-A). A maior indução de efeito citotóxico das águas coletadas no período de seca pode ser explicada pela baixa vazão dos rios, o que acarreta em uma maior concentração dos resíduos de cromo presentes nessas águas.

As análises dos índices mitóticos, realizadas com águas do rio Sapucaizinho e do Córrego dos Bagres, revelam que, em todos os tratamentos realizados, para as amostras coletadas no período de chuva de 2001 e 2002, não foram observadas diferenças significativas em relação ao controle negativo, exceto para o tratamento crônico, realizado com as amostras do Córrego dos Bagres. O controle positivo também apresentou diferença significativa em relação ao controle negativo. Esses resultados indicam que o cromo, realmente, pode ser considerado o agente tóxico dessas águas, capaz de promover mudanças no ciclo celular de *A. cepa*.

Durante o período de seca de 2001, foi observado um efeito citotóxico significativo para todos os tratamentos realizados com as amostras de água do Córrego dos Bagres. Já para o rio Sapucaizinho, não foi observada citotoxicidade para as amostras analisadas pelo tratamento contínuo. No tratamento agudo, porém, foi observada uma redução significativa dos

índices mitóticos para os pontos PB-T e PC. Esses pontos são os que apresentam altas concentrações de resíduos de cromo, por corresponderem aos locais de despejo do efluente do curtume, evidenciando que a presença de resíduo de cromo pode estar relacionada com a citotoxicidade das águas avaliadas. No tratamento crônico, a citotoxicidade foi observada para os pontos PA, PB-T e PC, que apresentaram valores significativamente diferentes do controle negativo. Por essa análise, o tratamento crônico revelou uma sensibilidade maior de avaliação da citotoxicidade do cromo, quando comparado com os resultados dos tratamentos contínuo e agudo. Esses resultados indicam que, no período de estiagem, quando a vazão do rio apresenta-se baixa, os resíduos de cromo podem ser, potencialmente, mais citotóxico para sistemas de *A. cepa*.

As águas do Córrego dos Bagres, coletadas no período de seca de 2002, apresentaram efeito citotóxico, observado em todos os tratamentos realizados. Os pontos com os mais baixos índices mitóticos foram FB e FC. No entanto, para as amostras de águas coletadas no rio Sapucaizinho, nesse mesmo período, houve uma redução dos índices mitóticos para todos os pontos investigados, com exceção do ponto PB-A (contínuo). Para o tratamento contínuo não foi possível observar diferenças significativas entre os pontos PB-T e PC e o controle positivo. Para o tratamento agudo, também não foi observada diferença significativa entre o ponto PB-T e o controle positivo. Esses resultados reforçam as indicações de que as águas dos

pontos com alta concentração de cromo total, segundo as análises químicas realizadas, apresentaram os maiores efeitos citotóxicos registrados nos testes realizados com *A. cepa*.

Para o tratamento realizado em água milli-Q, após o tratamento crônico (tratamento de recuperação), foi observada uma reversão nos índices mitóticos. Essa redução também foi observada para o tratamento de recuperação do controle positivo. Essa reversão do índice mitótico evidenciou a potencialidade de recuperação das células de *Allium cepa*, quando cessou a ação do cromo sobre elas. Como não houve continuidade na promoção de alterações do ciclo celular, após a volta das condições normais do meio, podemos sugerir que o cromo não apresenta potencial acumulativo de efeito citotóxico em células de *Allium cepa*.

De acordo com o observado em nossos estudos, podemos relacionar o efeito tóxico do efluente de curtume à presença do metal pesado cromo, uma vez que o controle positivo, realizado com solução aquosa de cromo III, responde igualmente às amostras de água coletadas em locais contaminados por esse metal.

Nossos dados revelam que o teste de índice mitótico de *Allium cepa* constitui um bom parâmetro de avaliação de citotoxicidade de ambientes aquáticos. Foi observado, em nossas análises, que todas as células submetidas aos tratamentos com amostras de águas contaminadas com altas concentrações de cromo tiveram uma redução dos seus índices

mitóticos. Esses resultados são coincidentes com os apresentados por Sahi et al. (1998), que investigaram a citotoxicidade de amostras de água de rios contaminados por cromo, na Índia. Os dados aqui apresentados corroboram também com as citações de Smaka-Kincl et al. (1997), que afirmam que agentes contaminantes da água podem alterar, reduzindo ou aumentando, os índices mitóticos das células meristemáticas de *A. cepa*.

9.4 – Análise do efeito genotóxico

Para as análises do efeito genotóxico das águas dos rios que recebem efluentes de curtume, foram escolhidos alguns organismos teste, (*Allium cepa*, *Oreochromis niloticus* e células de ovário de Hamster-CHO-K1), tidos, por vários autores, como organismos eficientes na avaliação de mutagênese ambiental.

O potencial de genotoxicidade das amostras de águas, coletadas em diferentes estações do ano de 2001 e 2002, foi avaliado pela freqüência de células aberrantes em meristemas de *A. cepa*, e pela freqüência de danos no DNA de *O. niloticus* (pela técnica de ensaio do cometa, micronúcleo e anormalidades nucleares) e no DNA de CHO-K1 (ensaio do cometa).

Dentre os organismos testes, temos que o *Allium cepa* constitui um ótimo organismo para a análise da genotoxicidade e, especificamente, da clastogenicidade. Segundo Rank e Nielsen, 1998; Grover e Kaur, 1999; Kong e Ma, 1999; Thierens et al., 1999; Haroun e Shehri, 2001; Patra e Sharma,

2002; Matsumoto et al., no prelo, *Allium cepa* apresenta cromossomos fáceis de serem observados devido ao tamanho que possuem e do baixo número que exibem em suas células somáticas.

Os danos induzidos por agentes genotóxicos são: quebras simples e duplas da cadeia principal do material genético; aberrações cromossômicas; formação de micronúcleo (MN); trocas de cromátides irmãs; além de erros nos processos de replicação do DNA e de expressão gênica (ZHITKOVICH et al., 1996; O'BRIEN et al., 2001; MATSUMOTO, 2003; MATSUMOTO et al., no prelo).

Foram realizadas análises em sistema teste de *Allium cepa* para avaliar a potencialidade de indução de efeito genotóxico de amostras de águas dos rios que recebem efluentes de curtume. Foram observados, por meio de células em divisão portadoras de anormalidades, os índices de aberrações cromossômicas de cada tratamento realizado, para todas as amostras de águas testadas.

Foi observada uma alta indução de aberrações cromossômicas nos meristemas radiculares de *A. cepa*, submetidas a germinação em águas contaminadas por cromo e em solução aquosa de cromo III (controle positivo). Esses resultados confirmam que o cromo apresenta um efeito genotóxico sobre células meristemáticas de *A. cepa*.

Embora a Legislação Estadual de São Paulo – Brasil (1995) estabeleça a necessidade de tratamento do efluente de curtume, antes do

despejo em ambientes aquáticos, para que se reduza concentração de cromo para 0.05 ppm, considerada não tóxica, observamos que concentrações mais baixas que as sugeridas para este metal já se mostraram eficientes para promoverem efeitos clastogênicos em organismos teste. Nossos dados são concordantes com os de Matsumoto et al. (no prelo), que descrevem que concentração de 0.01 mg/L de cromo induz efeito mutagênico em CHO-K1.

As aberrações mais freqüentes, observadas em nossas análises, foram: células com micronúcleo e binucleadas; células com aderências; quebras e perdas cromossômicas; c-metáfase; anáfases multipolares; anáfases com pontes. Nossos dados são semelhantes aos descritos por Grover e Kaur (1999) e Sudhakar et al., 2001, que afirmam a potencialidade do cromo em induzir essas mesmas aberrações cromossômicas em sistema teste de *Allium cepa*.

Para as análises do efeito genotóxico, das águas coletadas no período de chuva de 2001, realizados com sistema de *A. cepa,* foi observado que todos os pontos do rio Sapucaizinho não apresentaram diferenças significativas, para todos os tratamento, em relação ao controle negativo, evidenciando assim, nenhum efeito genotóxico nessas águas.

Para as amostras do Córrego dos Bagres, coletadas no período de chuva de 2001, o efeito genotóxico foi observado apenas para o tratamento onde as células meristemáticas permanecem por mais tempo em contato

com as águas testadas (tratamento crônico). Esse efeito foi observado, principalmente, para águas do ponto FB, que corresponde ao local do despejo do efluente do curtume, evidenciando, novamente, que o cromo deve ser a substância tóxica responsável pela clastogenicidade e genotoxicidade observada nos ensaios realizados com sistemas teste de *Allium cepa*. Esses dados reforçam as descrições de Grover e Kaur (1999) e Sudhakar et al. (2001), sobre a potencialidade do cromo em induzir efeitos clastogênicos em *Allium cepa*.

Para o rio Sapucaizinho foi observado, um efeito genotóxico em todas as amostras de água coletadas no período de chuva de 2002, para todos os tratamentos, com exceção do tratamento com água milli-Q, após o tratamento crônico (tratamento de recuperação). Neste teste foi observada uma recuperação dos efeitos clastogênicos das células de *A. cepa,* pois todos os tratamentos de recuperação realizados para esse período, apresentaram respostas de recuperação da normalidade do ciclo celular. A recuperação das condições normais do ciclo celular também foi observada nos testes de índice mitótico, já citadas anteriormente. Assim, o tratamento de recuperação parece mostrar a potencialidade de reversão de anormalidade de *A. cepa*, se o organismo exposto ao cromo voltar às condições de ambiente não poluído. Nossos dados sugerem que os efeitos clastogênicos, induzidos pelo cromo presente nas amostras de águas

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estudadas, não apresentaram um potencial cumulativo de mutagenicidade e clastogenicidade para sistemas de *A. cepa.*

Nossos dados são pioneiros quanto ao potencial de recuperação de danos promovidos pela ação do cromo sob células de *A. cepa*.

Para as amostras coletadas no período de seca de 2001, a maior freqüência de células meristemáticas com aberrações foi observada para os pontos PB-T, PB-L, PC e FB. Esses dados indicam a existência de substâncias tóxicas capazes de danificar o material genético e de agir durante o ciclo celular de *A. cepa*, resultando em aberrações cromossômicas. Foi observada uma alta freqüência de células com micronúcleo, células com pontes, quebras e perdas cromossômicas. Nossos dados são concordantes com os de Walker et al. (1996), Frieauff et al. (1998), Grover e Kaur (1999) e Patra e Sharma (2002), que descrevem a formação de micronúcleo como resultado do efeito clastogênico sobre os cromossomos, em resposta a agentes genotóxicos. Nossos dados também confirmam a potencialidade do metal pesado cromo induzir quebras simples ou duplas dos cromossomos, resultando em anormalidades na sua estrutura citadas por Von Burg e Liu, 1993; Stohs e Bagchi, 1995; Zhitkovich et al. 1996, Mount e Hockett, 2000, O'Brien et al., 2001, Matsumoto, 2003 e Matsumoto et al., no prelo.

Observamos, em nossas análises, a presença de células portadoras de pontes e quebras cromossômicas. As quebras cromossômicas são, possivelmente, decorrentes de pontes cromossômicas que se instalam em

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fases anteriores da divisão celular. Quando essas quebras acontecem, podem originar fragmentos duplicados em um dos pólos da célula, resultando em células desbalanceadas, quanto ao seu conteúdo genético. Segundo Fiskejö (1993) e Giacomelli (1999), as quebras cromossômicas podem estar diretamente relacionadas com a formação prévia de pontes ou aderências cromossômicas, decorrendo em fragmentos cromossômicos que originarão MN nas gerações celulares seguintes.

Em nossas análises, foram observadas células que sofreram interferência no fuso mitótico, resultando em perda de cromossomos inteiros, que persistiram até a telófase. Esses cromossomos, provavelmente, estarão também relacionados com a formação de células com micronúcleo, na próxima geração celular. Podemos sugerir que os resíduos de cromo, presentes nas amostras de água, interferiram no processo de polimerização dos fusos mitóticos, ocasionando segregação cromossômica errônea, durante o ciclo celular.

Segundo Fernandes (2002), além das perdas cromossômicas, a inativação no fuso mitótico, também pode ser comprovada pela presença de células em C-metáfases, em que não se observa a formação da placa equatorial organizada. Essa inativação levaria à formação de células com o dobro do número cromossômico, caracterizando as chamadas células poliplóides. Em nossas análises, embora tenha sido observada a presença de perdas cromossômicas, nenhuma célula poliplóide foi observada.

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Nossos dados revelam a presença de células com aderências cromossômicas. Assim, podemos sugerir que os resíduos de cromo possam ter impedido a migração correta dos cromossomos para os pólos, devido à sua ação aneugênica, o que promoveu um estacionamento da divisão celular em metáfase, devido à inativação do fuso mitótico dessas células.

Segundo Giacomelli (1999), a aderência cromossômica é um fenômeno citogenético amplamente descrito em plantas. Embora sua primeira descrição tenha sido feita por Köermicke, no início deste século, o termo "stickiness" foi empregado pela primeira vez em 1932, por Beadle, para descrever o aspecto pegajoso de cromossomos de milho, em células que haviam sofrido uma mutação recessiva. A presença de aderência cromossômica em células de *A. cepa,* submetidas às águas contaminadas pelo cromo, sugere a ocorrência de mutação nessas células.

Organismos aquáticos, como os peixes, acumulam poluentes diretamente, da água contaminada, e indiretamente, pela alimentação com outros organismos aquáticos contaminados. Os poluentes genotóxicos contaminam, além dos organismos aquáticos, todo o ecossistema e, finalmente, o homem, pelo processo de transferência de energia entre os níveis tróficos.

Foram realizadas análises dos efeitos genotóxicos pelo teste de MN; pela análise de anormalidades nucleares; pelo ensaio do cometa, utilizando peixe (*Oreochromis niloticus*) como organismo teste. Nossos dados revelam

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que todos os pontos avaliados do Córrego dos Bagres apresentaram efeitos genotóxicos, detectáveis por esses testes. A maior freqüência de danos foi observada para o ponto FB, que representa o local de despejo do efluente do curtume. Esses resultados reforçam o comprometimento desse ponto, quanto à sua contaminação pelo cromo utilizado nos processos de curtimento do couro de boi.

As anormalidades nucleares observadas são classificadas como núcleo "blebbed", "notched", "lobed" e "broken-eggs". Essas anormalidades, segundo Carrasco et al. (1990) e Çavas e Ergene-Gözukara (2003), são consideradas danos genotóxicos. A alta freqüência de anormalidades nucleares, observadas nos eritrócitos de peixes submetidos à amostra de água do ponto FB, evidenciou a potencialidade genotóxica dos resíduos de cromo presentes nesse local.

O ensaio do cometa tem sido proposto como uma ferramenta eficiente no biomonitoramento e mutagênese ambiental. Esse teste evidencia danos na molécula de DNA, pela migração em eletroforese (KLAUDE et al., 1996; ANDERSON e PLEWA, 1998; MITCHELMORE e CHIPMAN, 1998; LEE e STEINERT, 2003).

A técnica do ensaio do cometa, segundo Mitchelmore e Chipman (1998), é eficiente para os estudos de monitoramento de recursos hídricos, podendo utilizar peixe como organismo teste. As análises realizadas com o ensaio do cometa, revelaram que o efeito genotóxico foi significativo para as

águas dos pontos FA e FB, confirmando o efeito genotóxico do metal pesado cromo presente nas amostras investigadas.

Como o efeito genotóxico das águas do ponto FB foi observado para os três diferentes ensaios realizados (micronúcleo, anormalidades nucleares e cometa), podemos confirmar a atuação dos resíduos de cromo na indução de danos à estrutura do DNA. Esses resultados são concordantes com os de Lee e Steinert (2003), que afirmam que o teste de ensaio do cometa é até mais eficiente que o teste de MN para avaliação de genotoxicidade de organismos expostos.

Por meio de testes realizados com células de CHO-K1, *in vitro*, foi possível avaliar o efeito genotóxico das amostras de águas coletadas do Córrego dos Bagres, no ano de 2001, e no rio Sapucaizinho, no ano de 2002. Todos os pontos de coleta apresentaram danos no material genético, com valores superiores aos observados para o controle negativo, e inferiores aos observados para o controle positivo. O efeito genotóxico dessas águas foi mais intenso nos pontos FB e FC, para o córrego dos Bagres, e nos pontos PB e PC, para o rio Sapucaizinho. Os pontos PA e FA apresentaram efeitos genotóxicos menos intensos, pois esses pontos correspondem à montante do efluente, local que ainda não sofreu influência dos efluentes de curtume. Esses dados reafirmam que o efeito genotóxico observado foi realmente promovido pelo cromo, pois nos pontos onde a concentração do cromo total foi mais alta, observamos uma indução de genotoxicidade, enquanto que nos

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locais de baixa concentração desse metal, não houve indução de danos ao DNA.

Os dados referentes aos ensaios aqui apresentados são concordantes com os de Leonard e Lauwerys, 1980; Von Burg and Liu, 1993; Beyersmann et al., 1994; Stohs e Bagchi, 1995; Sahi et al., 1998; Singh et al., 1998; Mount e Hockett, 2000; Matsumoto, 2003, que descrevem que o cromo constitui um metal pesado com potencialidade citotóxica e genotóxica para plantas, animais e, conseqüentemente, para o homem.

10. – CONCLUSÃO

- O índice mitótico pode ser usado como um eficiente parâmetro para avaliação de mutagênese ambiental;

- As análises realizadas com amostras de água do Córrego dos Bagres mostraram que, para os pontos de coleta localizados após a emissão dos efluentes de curtume, apresentaram um efeito genotóxico para todas as estações do ano, sendo maior nos períodos de seca, quando a pluviosidade é sempre baixa neste ambiente;

- Análises de águas do rio Sapucaizinho não evidenciaram indução de efeito genotóxico para a estação chuvosa. Isto pode ser explicado pelo menor impacto sofrido, por esse rio, das atividades de curtumes, e pela alta pluviosidade neste período, o que promove uma maior diluição dos prováveis resíduos de cromo;

- Os índices pluviométricos influenciam nos efeitos de citotoxicidade, genotoxicidade e clastogenicidade de águas comprometidas com resíduos de cromo;

- Organismos teste submetidos aos tratamentos com águas contaminadas com cromo apresentaram uma maior indução de genotoxicidade em suas células;

- Águas derivadas de coletas em local de despejo de efluente de curtume, e imediatamente abaixo desse despejo, podem apresentar uma

maior potencialidade de indução de efeitos genotóxico em sistemas teste, devido à sua contaminação por cromo;

- Efeitos citotóxicos e genotóxicos em organismos teste já são perceptíveis para concentrações de 0.01 mg/L de cromo. Essa concentração é muito inferior à exigida pela Legislação do Estado de São Paulo (0.05mg/L);

- As concentrações de cromo permitidas para efluentes de curtume devem ser revistas, pois esse metal apresenta uma alta capacidade de promoção de danos em material genético.

11. – REFERÊNCIAS BIOBLIOGRÁFICAS

AI-SABTI, K. Clastogenic efects of five carcinogeniic chemicals on the cels of the common *Cyprinus carpio L.* Comp. **Biochemistry physiology,** [S.l.], v.85, 1986, p 5-9.

AL-SABTI, K.; METCALFE, C. Fish micrnuclei for assessing genotoxicity in water. **Mutation research**, Amsterdam, v.343, 1995, p.121-135.

AIYAR, J.; BORGES, K.M.; FLOYD, R.A.; WETTERHAHN, K.E. Role of Chromium(V), Glutathione Thiyl Radical And Hydroxyl Radical Intermediates In Chromium(Vi)-Induced Dna Damage. **Environmental and toxicology chemistry**, Pergamon, v.22, 1989, p.135-148.

ALLEMAN, M. A.; KOSTER, J.F.; WILSON, J.H.P.; EDIXHOVEN-BOSDIJK, A.; SLEE, R.G.; KROSS, M.J. E EIJK, H.G.V.The involvement of iron an lipid peroxidation in the pathogenesis of HCB-induced porphyria. Biochem. **Pharmacology**, Basel, v.34, 1985, p.161-166.

AMES, B. A.; SHINGENAGA, M.K.; PARK, E.M. Oxidative Damage and Repair: Chemical, Biological and Medical Aspects. Elmsford ed. Pergamon Press. New York, 1991.187 pp.

ANDERSON, D.; PLEWA, M.J. The International Comet Assay Workshop. **Mutagenesis**, [S.l.], v.13, 1998, p. 67-73.

AUST, S.D. Metal ions, oxygen radicals and tissue damage. **Bibliotheca nutritio et dieta**, Basel, v.43, 1989, p.266-277.

BAGCH, D.; STOHS, S.J.; DOWNS, B.W.; BAGCHI, M.; PREUSS, H.G. Cytotoxicity and oxidative mechanisms of differents forms of chromium. **Toxicology**, Limerick, v.180, 2002, p.5-22.

BEYERSMANN, D.; BLOCK, C.; MALVIYA, A. Effects of Cadmium on Nuclear Protein Kinase C. **Environmental health perspectives**, Research Triangle Parkj, v. 102, 1994, p.177-180.

BLAKELY, W.F.; FUCIARELLI, A.F.; WEGHER, B.J. E DIZDAROUGLU, M. Hydrogen peroxide-induced base damage in deoxyribonucleic- Acid. **Radiation research**, San Diego, v.12, 1990, p.338-343.

BLOCK, G. The data support a role for antioxidants in reducing cancer risk. **Nutrition reviews**, [S.l.], v.50, 1992, p.207-213.

BORGES, K.M.; BOSEWLL, J.S.; LIEBROSS, R.H.; WETTERHAHN, K.E. Activation of chromium (VI) by thiols results in chromium (V) formation chromium bing to DNA and altered DNA conformation. **Carcinogenesis**, Oxford, v.12, 1991, p.5551-5561.

BRIAT, J.F.; LEBRUN, M. Plan responses to metal toxicity. C.R. Acad. Sci. Paris. **Sciences de la vie**, Montrouge, v.322, 1999, 43-54.

CARRASCO, K.R.; TILBURY, K.L.; MAYERS, M.S. Assessment of the piscine micronuclei test as an *in situ* biological indicator of chemical contaminants effects. **Canadian journal of fisheries and aquatic sciences**, Ottawa, v. 47,1990, p. 2123-2136.

CHAKRAVARTY, B.; SRIVASTAVA, S. Toxicity of some heavy metals *in vivo* and *in vitro* in *Helianthus annuus.* **Mutation research**, Amsterdam, v.283, 1992, p.287-294.

CHAN, P.C.; PELLER, O.G.; KESNER, L. Copper(II)-catalyzed lipid peroxidation in liposomes and erythrocyte membrane. **Lipids**, Champaign, v.17, 1982, p331-337.

CODD, R.; DILLON, C.T.; LEVINA, A.; LAY, P.A. Studies on the genotoxicity of chromium: from the test tube to the cell. **Coordination chemistry reviews**, Amsterdam, v.216, 2001, p.537-582.

COHEN, M.D.; KARGACIN, B.; KLEIN, C.B.; COSTA, M. Mechanisms of chromium carcinogenicity and toxicity, **Critical riviews in toxicology**, Boca Raton, v.23, 1993, p.255-281.

CONNETT, P.H.; WETTERHAHN, K.E. Metabolism Of The Carcinogen Chromate By Cellular-Constituents. **Structure and bonding**, Berlin, v.54, 1983, p.93-124.

CONNETT, P.H.; WETTERHAHN, K.E. *In vitro* Reaction Of The Carcinogen Chromate With Cellular Thiols And Carboxylic-Acids. **Journal of the american chemical society**, [S.l.], v.107, n.14, 1985, p.4282-4288.

CONNOR, J.A.; FERGUNSON, M.A. **Essential medical genetics**. Smith Blackwell Scientific Publications. London, 260pp. 1993.

CUNNINGHAM, R.P. DNA repair: Caretakers of the genome? **Current biology**, London, v. 7, 1997, p. 576-579.

unesp

ÇAVAS, T.; ERGENE-GÖZÜKARA, S. Micronuclei, nuclear lesions and interphase silver-stained nucleolar organizer regions (AgNORs) as cyto-genotoxicity indicators in *Oreochromis niloticus* exposed to textile mill effluent. **Mutation research**, Amsterdam, v. 534, 2003, p. 93-99.

DASHWOOD, R.H.; BARILEY, G.S. Use of fish and fish transgenics in laboratory and field genotoxicology studies. **Mutation research**, Amsterdam, v. 399, 1998, p.123-124.

DE, S.K.; DEY, S.K.; ANDREWS, G.K. Cadmium teratogenicity and its relationship with metallothionein gene expression in midgestation mouse embryos. **Toxicology**, Limerick, v.64, 1990, p. 89-104.

DEBETTO P, TOSO RD, VAROTTO R, BIANCHI V, LUCIANI S. Effects Of Potassium Dichromate On Atp Content Of Mammalian-Cells Cultured In vitro. **Chemico-biological interactions**, Limerick, v.41, n.1, 1982, p.15-24.

DEBETTO, P.; LUCIANI S. Toxic Effect Of Chromium On Cellular-**Metabolism science of the total environmental**, [S.l.], v.71, n.3, 1988, p. 365-377.

DEPLEDGE, M.H. Genetic ecotoxicology: an overview. **Journal of experimental marine biology and ecology**, Amsterdam, v.200, 1996, p. 57-66.

FALK, G.; CATALAN. J.; NORPPA, H. Influence of Culture time on the frequency and contents of human lymphocyte micronuclei with and without cytochalasin B. **Mutation research**, Amsterdam, v.392, 1997, p. 71-9.

FALKENMARK, M.; ALLARD, B. Water-Quality and Disturbances of Natural freshwater. In: " The Handbook of Environmental Chemistry. Part A – Water Pollution". v. 5, Berlim, 1991, p. 46-78.

FERNANDES, T.C.C.A. Uso do teste de *Allium cepa* na detecção da toxicidade e genotoxicidade do herbicida trifluralina. Monografia (mestrado), Universidade Estadual Paulista, Rio Claro/SP, 2002, 44 pp.

FERRARI, I. Test do micronúcleo em cultura temporária de linfócitos. In: RABELLO-GAY, M.N.; RODRIGUES, M.A.L.R.; MONTELEONE-NETO, R. **Mutagênese, teratogênese e carcinogênese. Métodos e critérios de Avaliação**.,Revista Brasileira de Genética, Ribeirão Preto, 1991, p.107-112.

unesp

FISKEJO, G. The *Allium* test. As a standard in environmental monitoring. **Hereditas**, Lund, v. 102, 1985, p. 99-112.

FISKESJÖ, G. The *Allium cepa* in wastewater monitoring. **Environmental toxicology and water quality**: An International Jounal, v.8, 1993, p. 291-298.

FRIBERG, L.; NORDBERG, G.F.; VOUK, V.B. Handbook on the Toxicology of Metals., 2nd ed., Elsevier, Amsterdam. 1985.

FRIEAUFF, W.; POTTER-LOCHER, F.; CORDIER, A; SUTER, W. Automatic analysis of the *in vitro* micronucleus test on V79 cells. **Mutation research**, Amsterdam, v. 413, 1998, p. 57-68.

GIACOMELLI, F.R.B. Avaliação do comportamento meiótico em variedades de Aveia (*Avena sativa*) recomendadas para a região Sul. Dissertação (mestrado). Universidade Estadual de Maringá. Maringá-PR. 1999.

GONTIJO, A.M.M.C.; BARRETO, R. E.; SPEIT, G.; REYES, V. A. V.; VOLPATO, G. L.; SALVADORI, D. M. F. Anesthesia of fish with benzocaine does not interfere with comet assay results. **Mutation research**, Amsterdam, v. 534, 2003, p. 165-172.

GOMEZ-ARROYO, S.; VILLALOBOS-SPIETRINI, R. Chromosomal alterations induced by some chromium salts. **Cytologia**, Tokyo, v. 48, 1983, p. 185-203.

GROVER, I. S.; KAUR, S. Genotoxicity of wastewater samples from sewage and industrial effluent detected by the *Allium cepa* root anaphase aberration and micronucleus assays. **Mutation research**, Amsterdam, v. 426, 1999, p. 183-188.

HALLIWELL, B.; GUTTERIDGE, J.M.C. Oxygen radicals and the nervous system. **Trends neurosciences**, Amsterdam, v. 8, 1985, p-22-26.

HALLIWELL, B. How to characterize a biological antioxidant. **Free radical research communications,** [S.l.], v.9, 1990, p.1-32.

HALLIWELL, B.; AESCHBACH, R.; LOLIGER, J.; AUOMA, O.I. The chracterization of antioxidants. **Food and chemical toxicology**, Elmsford, v.33, n.7, 1995, p.601-617.

HOCHSTEIN, P.; ATALLAH, A.S. The nature of oxidants and antioxidants systems in the inhibition of mutation and cancer. **Mutation research**, Amsterdam, v.202, 1988, p.363-375.

HOROUN, S.A.; SHEHRI, A.M. Cytogenetic effects of *Calatropis procera* extract on *Vicia faba*L. **Cytologia**, Tokyo, v. 66, 2001, p. 373-378.

HOSE, J.E.; CROSS, J.N.; SMITH, S.G.; DIEHL, D. Elevated circulating erythrocyte micronuclei in fishes from contaminated sites of Sourthern California. **Marine environmental reserarch**, [S.l.], v. 22, 1987, p. 167-176.

HOUK, V.S. The genotoxicity of industrial wastes and effluents – a review. **Mutation research**, Amsterdam, v. 277, 1992, p. 91 – 138.

JONES, P.; KORTENKAMP, A.; O´BRIEN, P.; WANG, G.; YANG, G. Evidence for the generation of hydroxyl radicals from a chromium (V) intermediate isolated from the reaction of chromate with glutathione. **Archives of biochemistry and biophysics**, New York, v. 286, 1991, p-652-655.

KAWANISHI, S.; INOUE, S.; SANO, S. Mechanism of DNA cleavage induced by sodium chromate (VI) in the presence of hydrogen peroxide. **Journal of biological chemistry**, Bethesda, v. 261, 1986, p. 5952-5958.

KHORS, S.S.; PANDA, K.K.; PANDA, B.B. Genotoxicity of tetrodotoxin from buffer fish tested in root meristem cells of *Allium cepa* L. **Mutagenesis**, [S.l.], v.12, 1997, p. 265-9.

KLAUDE, M.; ERIKSSON, S.; NYGREN, J.; AHNSTRÖM, G. The comet assay: mechanisms and technical considerations. **Mutation research** Amsterdam, v.363, 1996, p. 89-96.

KNASMÜLLER, S.; GOTTMANN, E.; STEINKELLNER, H.; FOMIN, A.; PICKL, C.; PASCHKE, A.; GOD, R.; KUNDI, M. Detection of genotoxic effects of heavy metal contaminated soils with plant bioassays. **Mutation research**, Amsterdam, v.420, 1998, p.37-48.

KONG, M.S.; MA, T.H. Genotoxicity of contaminated soil and shallow well water detected by plant bioassays. **Mutation research**, Amsterdam, v. 426, 1999, p. 221-228.

KORTENKAMP, A.; CASADEVALL, M.; FAUX, S.P.; JENNER, A.; SHAYER, R.O.J.; WOODBRIDGE, N.; O´BRIEN, P. A role for molecular oxygen in the

formation of DNA damage during the reduction of the carcinogen chromium(VI) by glutathione. **Archives of biochemistry and biophysics**, New York, v. 329, n.2, 1996, p. 199-207.

KOVALCHUCK, O.; KOVALCHUCK, I.; ARKHIPOV, A., TELYUK, P.; HOHN, B.; KOVALCHUCK, I. The *Allium cepa* chromosome aberration test reliably measures genotoxicity of soil of inhabited areas in the Ukraine contaminated by the Chemobyl accident. **Mutation research**, Amsterdam, v. 415, v. 1-20, 1998, p. 47-57.

KRISHNAMOORTHY, C.R.; HARRIS, G.M. Equilibrium And Rate Study Of The Interaction Of Aqueous Chromium(III) Ion With Adenine. **Jounal of Coordination Chemistry**, [S.I.], v.10, 1980, p.55-65.

LEE, R.F.; STEINERT, S. Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. **Mutation research**, Amsterdam, v. 544, 2003, p.43-64.

LEONARD, A.; LAUWERYS, R.R. Carcinogenicity and mutagenicity of chromium. **Mutation research**, Amsterdam, v. 76, 1980, p. 227-239.

LI, Y.; TRUSH, M.A. Oxidation of hydroquinone by copper: Chemical mechanism and biological effects. **Archives of biophysics**, New york, v.300, 1993, p.346-355.

LIU, D.H.; JIANG, W.S.; LI, M.X. (1992) Effects of trivalent and hexavalent chromium on root-growth and cell-division of allium-cepa. **Hereditas**, Lund, v. 117, 1992, p. 23-29.

L´HUILLIER, L.; D´AUZAC, J.; DURAND, M.; MICHAUD-FERRIÈRE, N. Nickel effects on two maize (*Zea mays*) cultivars: growth, structure, Ni concentration, and localization. **Canadian journal of botany**, Ottawa, v.74, 1996, p.1547-1554.

MAGOS, L. Epidemiological and experimental aspects of metal carcinogenesis: physicochemical properties, kinetics, and the active species. **Environmental health perspectives**, Research Triangle Park, v.95, 1991, p. 157-189.

MAJER, B.J.; TSCHERKO, D.; PASCHKE, A; WENNRICH, R.; KUNDI, M.; KANDELER, E.; KNASMÜLLER, S. Effects of heavy metal contamination of soils on micronucleus induction in *Tradescantia* and on microbial enzyme

activities: a comparative investigation. **Mutation research**, Amsterdam, v.515, 2002, p.111-124.

MATSUMOTO, S.T. MALAGUTI, M.; FONSECA, I.C.; MARIN-MORALES, M.A. Avaliação genotóxica de um Córrego, Município de Franca/SP, que recebe efluente de curtume, utilizando o *Allium cepa* como organismo teste. **Genetics and molecular biology**, Riobeirão Preto, Supplement. v.26, 2003, p.200.

MATSUMOTO, S.T.; MANTOVANI, M.S.; MALAGUTI, M.; MARIN-MORALES, M.A. Investigation Of The Genotoxic Potential Of The Waters Of A River Receiving Tannery Effluents By Means Of The *In Vitro* Comet Assay. . **Cytologia**, no prelo, Tokyo.

MATSUMOTO, S. T.; (2003) Efeitos tóxicos e genotóxicos de metais pesados, especificamente do cromo trivalente e hexavalente. Monografia (doutorado) Universidade Estadual Paulista. São José do Rio Preto-SP.

McCARTHY JF, JACOBSON DN, SHUGART LR, JIMENEZ BD. Preexposure To 3-Methylcholanthrene Increases Benzo[A]Pyrene Adducts On Dna Of Bluegill Sunfish. **Marine environmental research**, Barking, v.28, n.1-4, 1989, p.323-328.

METCALFE, C.D. Induction of micronuclei and nuclear abnormalities in the erythrocytes of mudminnows and brow bullheads. **Bulletim Environmental Contam Toxicology**, [S.l.], v. 40, 1988, p. 489-495.

MINISSI, S.; LOMBI, E. Heavy metal content and mutagenic activity, evaluated by *Vicia faba* micronucleus test, of Tiber river sediments. **Mutation research**, Amsterdam, v.39 1997, p.317-321.

MINOTTI, G.; AUST, S.D. The role of iron in the initiation of lipid peroxidation. **Chemistry and physics of lipids**, Limerick, v.44, 1987, p.191-208.

MITCHELMORE, C.L.; CHIPMAN, J.K. DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. **Mutation research**, Amsterdam, v.399, 1998, p.135-147.

MOLYNEUX, M.J.; DAVIES, M.J. Direct Evidence For Hydroxyl Radical-Induced Damage To Nucleic-Acids By Chromium(Vi)-Derived Species - Implications For Chromium Carcinogenesis. **Carcinogenesis**, Oxford, v.16, n.4, 1995, p.875-882.

unesp

MORAES, D.S.L. Avaliação dos potenciais tóxicos, citotóxicos e genotóxicos de águas ambientais de Corumbá-MS em raízes de *Allium cepa*. Dissertação, para obtenção do título de mestre junto ao Curso de Genética e Melhoramento da UEL – Londrina/Pr. 158p., 2000.

MORAES, D.S.D.; JORDÃO, B.Q. Evaluation of the genotoxic potential of municipal waste water discharged into the Paraguay river during periods of flood and drought. **Environmental toxicology**, New York, v. 16, 2001, p. 113-116.

MOUNT, D.R.; HOCKETT, J.R. Use of toxicity identification evaluation methods to characterize, identify, and confirm hexavalent chromium toxicity in an industrial effluent. **Water research**, [S.l.], v.34, n.4, 2000, p.1379-1385.

NATARAJAN, A.T.; OBE, G. Mutagenicity testing with cultured mammalian cells: Citogenetic Assays in Mutagenicity New Horizons in Genetic Toxicology. Ed. By J.A. Heddle**.** Academic Press, New York, 1982, 213pp.

OBE, G.; NATARAJAN, A.T.; PALITTI, F. Role of DNA doublestrand breaks in the formation of radiation – induced chromosomal aberrations. Progress in **Mutation research**, Amsterdam, v. 4, 1982, p.1-9.

O´BRIEN, P.; KORTENKAMP, A. The chemistry underlying chromate toxicity **Transition metal chemistry**, Weinheim, v.20, n.6, 1995, p.636-642.

O'BRIEN, T.; XU, J.; PATIERNO, S.R. Effects of glutathione on chromium-induced DNA crosslinking and DNA polymerase arrest. **Molecular and cellular biochemistry,** The Hague, v. 222, 2001, p.173-182.

OHBA, H.; SUKETA, Y.; OKADA, S. Enhancement Of *In vitro* Ribonucleic-Acid Synthesis On Chromium(III)-Bound Chromatin. **Journal of inorganic biochemistry**, New York, v.27, n.3, 1986, p.179-189.

OHE, T.; WHITE, P. A.; DeMARINI, D.M. Mutagenic characteristics of river waters flowing through large metropolitan areas in North America. **Mutation research**, Amsterdam, v. 534, 2003, p. 101-112.

OKADA, S.; OHBA, H.; TANIYAMA, M. Alterations In Ribonucleic-Acid Synthesis By Chromium(III). **Journal of inorganic biochemistry**, New York, v.15, 1981, p. 223-231.

unesp

OKADA, S.; TSUKADA, H.; SUZUKI, M.; OHBA, H. Effect Of Chromium(III) On RNA-Synthesis In Mouse-Liver And Regenerating Rat-Liver. **Journal of toxicology and environmental health**, New York, v.29, 1983, p.45-45.

OLIVE, P.L.; WLODEK, D.; DURAND, R.E.; BANÁTH, P. Factors influencing DNA Migration from individual Cells Subjected to Gel Electrophoresis. **Experimental cell research**, New York, v.198, 1992, p.259-267.

OZAWA, T.; HANAKI, A. Spin-Trapping studies on the reactions of Cr(III) with hudrogen peroxide in the presence of biological reductants: Is Cr(III) nontoxic? **Biochemistry international**, Marrickville, v.22, 1990, p-343-352.

OZAWA, T.; UEDA, J.; SHIMAZU, Y. DNA single strand breakage by copper (II) complexes and hydrogen peroxide at physiological conditions. **Biochemistry and molecular biology international**, Marrickville, v.31, 1993, p.455-461.

PASCALICCHIO, A.E. Contaminação por metais pesados. Ed. Annablume, São Paulo/SP, 2002, 132pp.

PATRA, M.; SHARMA, A. Relative efficacy of *Allium cepa* and *Allium sativum* in anaphase-telophase test screening metal genotoxicity. **Biologia**, [S.l.], v. 57, 2002, p. 409-414.

PAVLICA, M.; BESENDORFER. V.; ROSA, J.; PAES, D. The cytotoxic effect of wastewater from the phosphoric gypsum depot on common oak (*Quercus robur* L.) and shallot (*Allium cepa* var. *ascalonicum)*. **Chemosphere**, Oxford, v. 41, 2000, p.1519-27,

PRESTON, R.J.; DEAN, B.J.; GALLOWAY, S.; HOLDEN, H.; McFEE, A.F.; SHELBY, M. Mammalian *in vivo* cytogenetic assay. Analysis of chromosome aberration in bone marrow cells. **Mutation research**, Amsterdam, v.189, 1987, p.157-165.

PRYOR, W. A. Why is the hydroxyl radical the only radical that commonly adds to DNA? Hypothesis: it is a rare combination of high electrophiliciity, high thermochemical reactivity, and a mode of production that occurs near DNA. **Free radical biology and medicine**, Oxford, v.4, 1988, p.219-223.

QUINZANI-JORDÃO, B. Ciclo celular em meristemas. La formatión de intercâmbios entre cromátidas hermanas. Tese de Doutorado, Universidade de Complutense, Madrid, 276p. 1987.

RABELO-GAY, M. N.; RODRIGUES, M.A.L.R.; MONTELEONE-NETO, R.(Ed.) Mutagênese, Teratogênese e Carcinogênese: Métodos e critérios de avaliação. Ribeirão Preto: Sociedade Brasileira de Genética/Revista Brasileira de Genética, 1991.

RANK, J.; NIELSEN, M.H. *Allium cepa* anaphase-telophase root tip chromosome aberration assay on N-methyl-N-nitrosourea, Maleic hydrazid, Sodium azide and ethyl methanesulfonate. **Mutation research**, Amsterdam, v. 390, 1997, p. 121-27.

RANK, J.; NIELSEN, M. H. Genotoxicity testing of wastewater sludge using the Allium cepa anaphase-telophase chromosome aberration assay. **Mutation research**, Amsterdam, v. 418, 1998, p. 113-119.

RAJAGURU, P.; VIDYA, L.; BASKARASETHUPATHI, B.; KUMAR, P.A.; PALANIVEL, M.; KALAISELVI, K. Genotoxicity evaluation of polluted ground water in human peripheral blood lymphocytes using the comet assay. **Mutation research**, Amsterdam, v. 517, 2002, p. 29-37.

ROBERTSON, A.L. The poisoning of roots of *Zea mays* by nickel ions, and the protection afforded by magnesium and calcium. **New phytology**, [S.l.], v.100, 1985, p.173-189.

ROJAS, E.; HERRERA, L.A.; POIRIER, L.A.; OSTROSKY-EWGMAN, P. Are metals dietary carcinogens? **Mutation research**, Amsterdam, v.443, 1999, p.157-181.

ROSSMAN, T.G. Metal mutagenesis, *in:* GOYER, R.A.; CHERIAN, M.G. Toxicology of Metals-Biochemical Aspects, Ed. Springer, New York, 1994, 405pp.

RYAN, T.; AUST, S.D. The role of iron in oxygen-mediated toxicities. **Critical reviews in toxicology**, Roca Raton, v.22, 1992, p.119-141.

SAHI, A.N.; SINGH, S.K.; SEM, P.K.; SINGH, R.N. (1998) Gytogenetic response of hexavalent shromium-induced somatic cell abnormalities in *Allium cepa*, **Cytobios**, Cambridge, v. 96, 1998, p. 71-79

SCHAAPER, R.M.; KOPLITZ, R.M.; TKESHELASHVILI, L.K.; LOEB, L.A. Metal-Induced Lethality And Mutagenesis - Possible Role Of Apurinic Intermediates. **Mutation research**, Amsterdam, v.177, 1987, p.179-188.

unesp

SCHOLZ, R.W.; GRAHAM, K.S.; WYNN, M.K. Interaction of glutathione and α- tocapherol in the inhibition of lipid peroxidation of rat liver microsomes. *In:* REDDY, C.C.; HAMILTON, G.A. Biological Oxidation Systems, Ed. Madyastha, v.2. Academic Press, San Diego, London, 1990. 867pp.

SIES, H. Strategies Of Antioxidant Defense. **European Journal Of Biochemistry**, Berlin, v.215, n.2, 1993, p.213-219.

SINGH, J.; BRIDGEWATER, L.C.; PATIERNO, S.R. Differential sensitivity of chromium-mediated DNA interstrand crosslinks and DNA-protein crosslinks to disruption by alkali and EDTA. **Toxicology sciences** [S.l.], v.45, n.1, 1998, p.72-76.

SMAKA-KINCL, V.; STEGNAR, P.; LOUKA, M.; TOMAN, M.J. The evaluation de waste, surface and ground water quality using the *Allium* test procedure. **Mutation research**, Amsterdam, v.368, 1997, p. 171-79.

SNOW, E.T. Metal carcinogenesis: mechanistic implications. **Pharmacology and therapeutics**, Oxford, v.53, 1992, p.31-65.

SNOW, E.T.; XU, L. Chromium (III) bound to DNA templates promotes increased polymerase processivity and decreased fidelity during replication *in vitro*. **Biochemistry**, New York, v.30, n.47, 1991, p.11238-11245.

STADTMAN, E.R. Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalysed reactions. **Annual review of biochemistry**, Palo Alto, v.62, 1993, p.797-821.

STEINKELLNER, H.; MUN-SIK, K.; HELMA, C.; ECKER, S.; MA, T.H.; HORAK, O; KUNDI, M; KNASMULLER, S. Genotoxic effects of heavy metal: comparative investigation with plant bioassays. **Environmental mutagenesis**, New York, v.31, 1998, p.183-191.

STOHS, S.J.; BAGCHI, D. Oxidative mechanisms in the toxicity of metal ions. **Free radical biology medical** [S.l.], v.18, n.2, 1995, p. 321-336.

SUDHAKAR, R.; NINGE-GOWDA, K.N.; VENU,G. Mitotic abnormalities induced by silk dyeing industry effluents in the cells of *Allium cepa.* **Cytologia**, Tokyo, v. 66, 2001, p. 235-239.

SUGDEN, K.D.; BURRIS, R.B.; ROGERS, S.J. An Oxygen Dependence In Chromium Mutagenesis. **Mutation research**, Amsterdam, v.244, 1990, p.239-244.

SUGIYAMA, M.; TSUZUKI, K.; HARAMAKI, N. DNA single-strand breaks and cytotoxicity induced by sodium chromate (VI) *in* Xu,, J.; PATIERNO, S.; Bogdanffy, M. Interlaboratory validation of a new assay for DNA-protein crosslinks. **Mutation research**, Amsterdam, v.369, 1996, p.13-21.

SUNDERMAN JR, F.W. Recent advances in metal carcinogenesis. **Annalis of clinical and laboratory science**, [S.l.], v.14, 1984, p.93-122.

SUNDERMAN JR., F.W.; PLOWMAN, M.C.; KROFTOVA, S.; GRBAC-IVANKOVIC, S.; FOGLIA, L.; CRIVELLO, J.F. Effects of teratogenic exposure to Zn2+, Cd2+, Ni2+, Co2+ and Cu2+ on metallothionein content and metallothionein-mRNA contents of *Xenopus*embryos. **Pharmacology and toxicology**, Copenhagen, v.76, 1995, p.178-184.

TKESHELASHVILI, L.K.; SHEARMAN, C.W.; ZAKOUR, R.A.; KOPLITZ, R.M,; LOEB, L.A. Effects Of Arsenic, Selenium, And Chromium on The Fidelity Of DNA-Synthesis. **Cancer research**, Baltimorem, v.40, n.7, 1980, p.2455-2460.

THIERENS, H.; VRAL, A.; BARBE, M.; AOUSALAH, B.; DeRIDDER, L. A cytogenetic study of nuclear power plant workers using the micronucleus-centromere assay. **Mutation research**, Amsterdam, v. 445, 1999, p. 105-111.

TSAPAKOS, M.J.; WETTERHAHN, K.E. The Interaction Of Chromium With Nucleic-Acids. **Chemico-biological interactions**, Limerick, v.46, 1983, p.265-277.

VOITKUN, V.; ZHITKOVICH, A.; COSTA, M. Complexing Of Amino-Acids To DNA By Chromate In Intact-Cells. **Environmental health perspectives**, Research Triangle Park**,** v.102, 1994, p. 251-255.

VON BURG, R.; LIU, D. Chromium and hexavalent chromium. **Journal of applied toxicology**, Chichester, v.13, 1993, p.225-230.

WALKER, J. A.; BOREHAM, D.R.; UNRAU, P.; DUCAN, A.M.V. Chromosome content and ultrastructure of radiation – induced micronuclei. **Mutagenesis**, [S.l.],v.11, n. 5,1996, p. 419-24.

cm 1 2 3 4 5 6 7 8 unesp 12 13 14 15 16 17 18

WARREN, G.; SCHULTZ, P.; BANCROFT, D.; BENNETT, K.; ABBOTT, E.T.; ROGER, S.; Mutagenicity of a series of hexacoordinate chromium (III) compounds. **Mutation research**, Amsterdam, v.90, 1981, p.111-118.

WHITE, P.A.; RASMUSSEN, J.B.; BLAISE, C. Comparing the presence, potency, and potencial hazard of genotoxins extracted from a broad range of Instrial effluents. **Environmental and molecular mutagenesis**, New York, v.27, 1996, p. 116-39.

WOLF, T.; KASEMANN, R.; Ottenwalder, H. Molecular Interaction Of Different Chromium Species With Nucleotides and Nucleic-Acids. **Carcinogenesis,** Oxford, v.10, 1989, p.655-659.

WÜRGLER, F.E.; KRAMERS, P.G. Environmental effects of genotoxins (eco-genotoxicology). **Mutagenesis**, [S.l.], v.7, 1992, p.321-327.

ZHITKOVICH, A.; VOITKUN, V.; COSTA, M. Formation of the amino acid-DNA complexes by hexavalent and trivalent chromium *in vitro*: Importance of trivalent chromium and the phosphate group. **Biochemistry**, New York, v.35, 1996, p.7275-7282.

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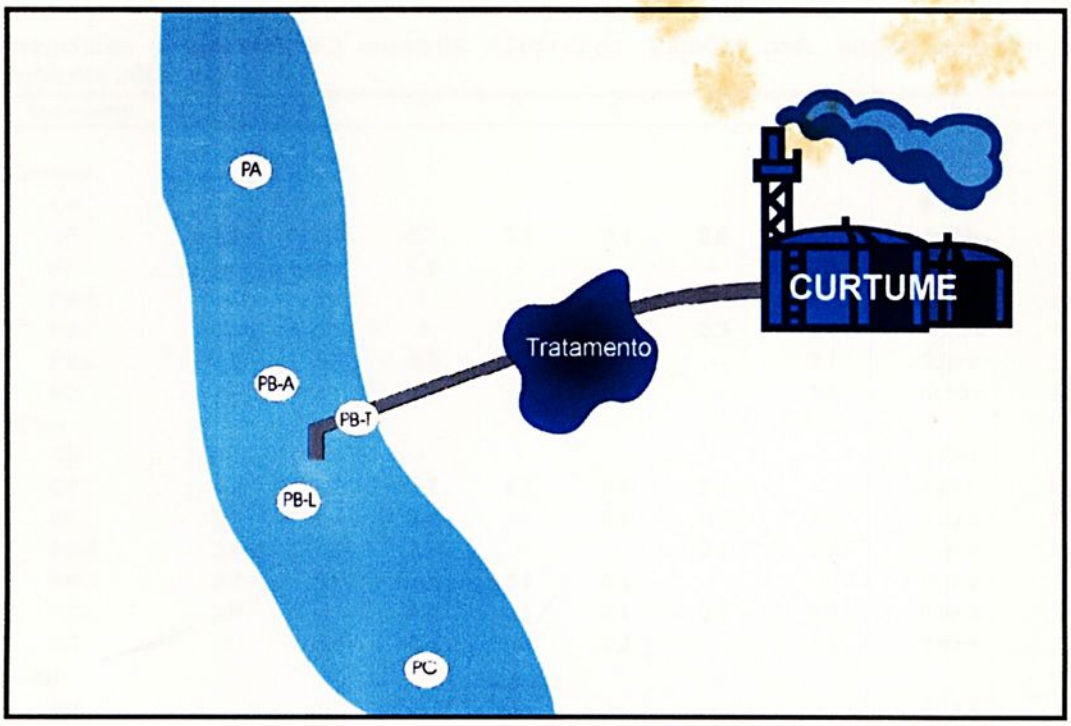
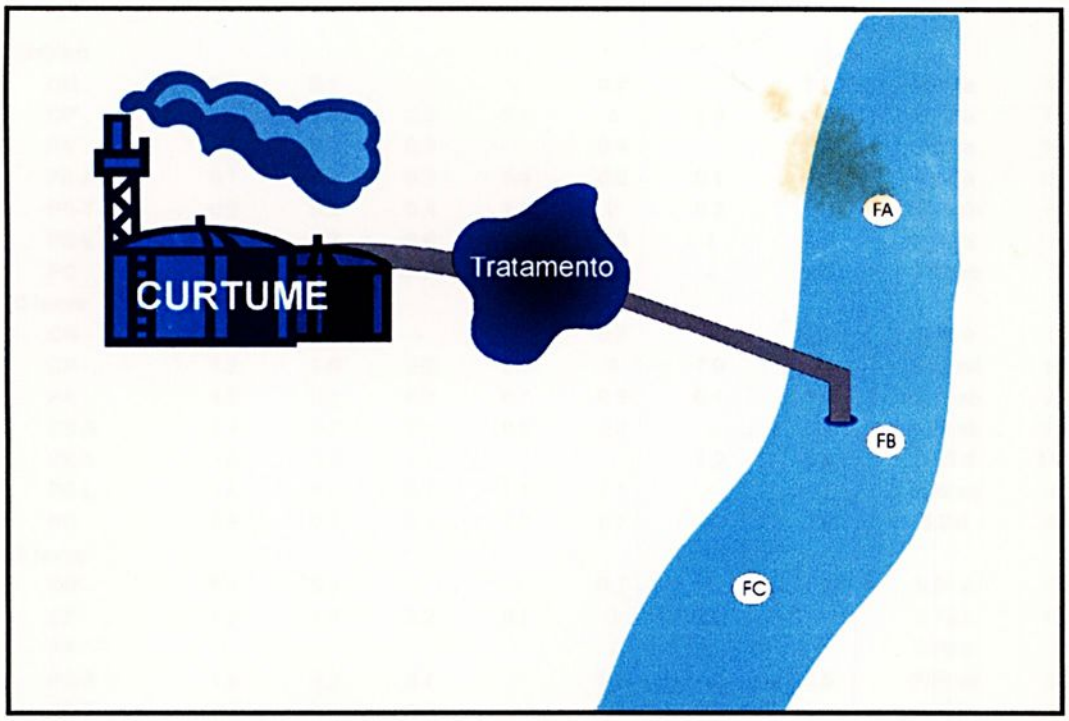
Pontos de coleta no rio Sapucaizinho, Município de Patrocínio Paulista/SP.

ANEXOS

Pontos de coleta em Córrego dos Bagres, Município de Franca/SP.



Pontos de coleta no rio Sapucaizinho, Município de Patrocínio Paulista/SP.



Pontos de coleta no Córrego dos Bagres, Município de Franca/SP.

Células irregulares observadas em raízes de *Allium cepa* tratadas com águas coletadas no rio Sapucaizinho em 2001.

período	Tratamento	A	B	C	D	E	F	G	H	I
chuvoso										
	Contínuo	-								
	CN	-	-	-	-	-	-	-	9.75 a	0.0 a
	CP	0.3	1.2	0.7	0.2	0.4	2.8	-	4.97 b	10.95 b
	PA	1	0.9	0.9	-	-	-	0.8	8.95 a	1.75 a
	PB-A	1.1	0.9	1	-	-	0.3	2.2	8.49 a	0.82 a
	PB-T	1	1	1	-	-	0.3	1.3	10.76 a	1.29 a
	PB-L	1.5	0.9	0.5	-	-	-	2.1	9.50 a	1.25 a
	PC	1	1.1	0.6	-	0.1	-	1.8	10.50 a	1.45 a
	20 hrs									
	CN	-	-	-	-	-	-	-	9.75 a	0.0 a
	CP	0.3	1.2	0.7	0.2	0.6	2.8	-	4.97 b	10.95 b
	PA	0.6	0.4	0.4	-	0.1	0.7	0.2	8.62 a	2.72 a
	PB-A	2.1	0.25	0.1	-	-	0.1	0.3	7.07 a	2.20 a
	PB-T	0.7	0.2	0.1	0.1	0.1	-	1	6.33 a	1.96 a
	PB-L	1.7	0.2	0.2	0.1	0.1	0.2	0.8	8.89 a	1.35 a
	PC	1	0.5	0.4	-	0.2	-	1.1	9.43 a	2.22 a
	72 hrs									
	CN	-	-	-	-	-	-	-	9.75 a	0.0 a
	CP	0.3	1.2	0.7	0.2	0.6	2.8	-	4.97 b	10.95 b
	PA	0.3	0.3	0.2	-	-	-	0.5	8.91 a	0.55 a
	PB-A	0.4	0.4	0.2	-	-	0.1	0.7	8.34 a	0.71 a
	PB-T	1	0.2	0.1	-	-	-	0.3	4.93 a	0.50 a
	PB-L	0.9	0.3	0.2	0.4	-	0.1	1	7.18 a	1.60 a
	PC	0.2	0.1	-	-	-	-	0.1	5.37 a	0.47 a
seca										
	Contínuo									
	CN	0.6	0.1	-	-	0.7	-	1.2	8.91 a	0.52 c
	CP	1.2	1.8	2.2	0.1	3	7.9	-	5.72 b	18.07 a
	PA	0.7	0.2	0.3		0.4	-	0.9	8.62 a	1.61 bc
	PB-A	0.7	0.4	0.3	0.4	0.3	0.1	1.5	8.92 a	3.16 b
	PB-T	0.6	0.2	0.4	1.1	0	0.2	2.3	6.86 ab	6.06 b
	PB-L	3.1	0.7	0.6	1.8	0.3	1	4.8	8.82 a	3.85 b
	PC	0.9	0.5	0.31	0.5	0.7	-	2.3	7.62 ab	3.03 b
	20 horas									
	CN	0.6	0.1	-	-	0.7	-	1.2	8.91 a	0.52 c
	CP	1.2	1.8	2.2	0.1	3	7.9	-	5.72 cd	18.07 a
	PA	1.5	0.2	0.3	0.7	0.6	0.1	1.9	8.11 ab	2.06 bc
	PB-A	1.4	0.2	0.1	0.8	0.2	-	1.4	8.59 ab	1.52 bc
	PB-T	7.6	0.1	4.1	-	-	1.2	5.4	2.95 d	11.78 bc
	PB-L	1.6	0.5	0.7	1.7	1.3	-	4.2	8.39 ab	4.84 b
	PC	0.8	0.3	0.4	1.7	0.7	-	2.1	5.9 d	2.99 b
	72 horas									
	CN	0.6	0.1	-	-	0.7	-	1.2	8.91 a	0.52 a
	CP	1.2	1.8	2.2	0.1	3	7.9	-	5.72 b	18.07 b
	PA	-	-	-	-	-	-		5.68 b	0.0 a
	PB-A	1.6	0.2	0.1	-	0.2	-	0.5	6.69 ab	0.70 a
	PB-T	2.3	0.4	0.6	-	-	0.1	1.1	5.32 b	1.93 a
	PB-L	1.2	0.2	0.32	0.1	0.4	-	1	6.48 ab	1.56 a
	PC	1.5	0.1	0.3	0.7	0.6	0.1	1.8	5.97 b	2.58 a

A- Micronúcleo; B- Anáfase com ponte; C- Anáfase multipolar; D- Atraso anafásico; E- Somatória de quebra e perda cromossômica; F- Somatória de C-metáfase e Aderência; G- Célula binucleada; H-Freqüência do índice mitótico; I- Freqüência de células aberrantes em relação á células em divisão. Médias seguidas pela mesma letra não diferem entre si no nível de significância de 5% pelo teste de Kuskal-Wallis feito para os postos médios.

unesp

Células irregulares observadas em raízes de *Allium cepa* tratadas com águas coletadas no rio Sapucaizinho em 2002.

período	Tratamento	A	B	C	D	E	F	G	H	I
chuvoso										
	Contínuo									
	CN	2	-	-	-	0.9	1.0	-	8.29 a	0.18 d
	CP	1.8	1.6	2.0	-	2.6	5.4	-	1.43 b	15.81 a
	PA	0.3	0.4	0.2	0.2	0.5	1.2	0.2	11.73 a	3.66 c
	PB-A	0.6	0.4	0.5	-	1.2	1.6	0.4	8.05 a	5.30 bc
	PB-T	0.5	0.7	0.2	0.1	1.7	3.2	0.1	7.02 a	9.33 ab
	PB-L	0.4	0.6	0.1	0.3	0.7	1.8	0.1	8.49 a	4.68 bc
	PC	0.1	-	-	-	0.6	1.3	-	6.66 a	2.89 c
	20 hrs									
	CN	2	-	-	-	0.9	0.1	-	8.29 a	0.18 c
	CP	1.8	1.6	2.0	-	2.6	5.4	-	1.43 b	15.81 a
	PA	0.5	0.4	0.6	0.1	0.4	1.5	-	6.56 a	4.85 b
	PB-A	0.4	2.5	0.9	-	0.4	1.8	0.1	7.65 a	5.88 b
	PB-T	0.8	-	-	-	-	0.3	0.9	2.64 a	2.57 bc
	PB-L	0.2	0.8	0.3	0.1	0.7	2.0	0.1	7.31 a	5.83 b
	PC	0.2	0.4	0.2	-	0.1	1.0	0.3	7.5 a	3.00 bc
	72 hrs									
	CN	2	-	-	-	0.9	0.1	-	8.29 a	0.18 d
	CP	1.8	1.6	2.0	-	2.6	5.4	-	1.43 b	15.81 a
	PA	0.4	0.3	-	-	0.9	2.6	0.2	5.81 a	6.49 bc
	PB-A	0.4	0.3	0.1	0.1	0.5	1.1	0.6	6.38 a	2.65 cd
	PB-T	2.0	0.6	0.7	0.1	0.6	2.8	0.3	5.22 a	9.64 ab
	PB-L	0.7	1.0	0.6	0.1	0.9	1.9	0.1	6.11 a	8.51 ab
	PC	0.6	0.2	0.2	-	0.1	1.2	-	5.82 a	3.67 bc
	Recuperação									
	CN	2	-	-	-	0.9	0.1	-	8.29 a	0.18 c
	CP	1.8	1.6	2.0	-	2.6	5.4	-	1.43 a	15.81 a
	PA	0.5	-	-	-	0.7	2.1	-	4.7 a	6.84 b
	PB-A	0.8	0.1	0.3	0.2	1.1	0.6	-	5.03 a	4.92 b
	PB-T	2.0	0.4	0.2	0.2	0.9	1.0	-	4.97 a	5.08 b
	PB-L	-	0.1	-	-	0.2	0.2	-	2.21 a	4.02 bc
	PC	0.9	0.5	0.4	0.1	0.3	1.9	0.3	4.33 a	8.23 ab
seca										
	Contínuo									
	CN	2.2	1.6	-	-	-	-	-	11.36 a	2.04 c
	CP	4.4	2.2	0.8	0.9	1	5.1	0.1	6.81 b	14.42 a
	PA	2.0	1.0	0.3	0.4	0.7	2.4	0.1	8.24 a	7.37 bc
	PB-A	1.1	0.3	0.2	0.1	1.5	1.5	0.1	7.64 a	5.17 b
	PB-T	1.1	0.6	1.0	0.3	2.1	1.4	0.1	7.74 ab	5.65 b
	PB-L	1.3	1.5	1.1	0.2	1.2	0.8	0.3	7.54 a	5.62 b
	PC	1.4	0.4	0.2	0.1	1.0	1.0	0.3	7.47 ab	3.94 b
	20 horas									
	CN	2.2	1.6	-	-	-	-	-	11.36 a	2.04 c
	CP	4.4	2.2	0.8	0.9	1.0	5.1	0.1	6.81 cd	14.42 a
	PA	1.2	0.5	0.2	-	1.6	2.1	0.2	5.56 ab	5.85 bc
	PB-A	0.2	1.1	0.8	-	2.8	3.8	-	7.17 ab	6.32 bc
	PB-T	0.6	0.4	0.4	-	0.1	2.3	0.4	4.21 d	4.32 bc
	PB-L	0.4	1.9	0.9	-	0.8	2.1	-	6.45 ab	5.22 b
	PC	1.0	1.1	0.8	-	1.5	2.7	0.2	7.58 bc	5.61 b
	72 horas									
	CN	2.2	1.6	-	-	-	-	-	11.36 a	2.04 b
	CP	4.4	2.2	0.8	0.9	1.0	5.1	0.1	6.81 b	14.42 a
	PA	1.1	0.5	1.2	0.2	0.3	3.4	0.1	7.17 b	5.52 b
	PB-A	1.2	1.0	0.2	-	1.4	3.7	0.1	7.21 b	5.65 b
	PB-T	3.6	1.2	0.6	-	1.2	1.5	0.3	4.87 b	9.23 b
	PB-L	1.3	2.3	0.9	-	3.1	3.3	-	7.31 ab	8.42 b
	PC	1.2	0.3	0.6	0.4	2.1	6.1	-	6.39 b	9.62 b
	Recuperação									
	CN	2.2	1.6	-	-	-	-	-	11.36 a	2.04 c
	CP	4.4	2.2	0.8	0.9	1.0	5.1	0.1	6.81 a	14.42 a
	PA	1.8	0.5	0.3	0.1	1.9	1.6	0.6	4.53 a	4.82 c
	PB-A	0.9	-	0.2	0.2	1.4	0.3	-	6.26 a	3.57 c
	PB-T	2.1	1.1	0.6	1.4	2.1	4.3	0.3	6.43 a	11.69 ab
	PB-L	1.5	1.6	1.4	0.4	4.4	2.9	0.2	6.42 a	11.72 ab
	PC	1.8	1.0	0.7	0.2	2.9	2.9	-	3.92 a	8.38 bc

A- Micronúcleo; B- Anáfase com ponte; C- Anáfase multipolar; D- Atraso anafásico; E- Somatória de quebra e perda cromossômica; F- Somatória de C-metáfase e Aderência; G- Célula binucleada; H-Freqüência do índice mitótico; I- Freqüência de células aberrantes em relação á células em divisão. Médias seguidas pela mesma letra não diferem entre si no nível de significância de 5% pelo teste de Kuskal-Wallis feito para os postos médios.

unesp

Células irregulares observadas em raízes de *Allium cepa* tratadas com águas coletadas no rio Córrego dos Bagres em 2001.

Período	tratamento	A	B	C	D	E	F	G	H	I	J	K
seca												
	contínuo											
	CN	0.6	0.1	-	-	-	0.7	-	-	1.2	8.91 a	0.52 c
	CP	1.2	1.8	2.2	0.1	1	2	5.2	2.7	-	5.72 b	18.07 a
	FA	1.1	0.4	0.3	0.4	-	0.6	-	-	1.7	6.34 b	2.61 bc
	FB	0.7	0.4	0.7	0.4	-	0.5	0.1	0.2	2.3	7.19 ab	3.23 b
	FC	0.8	0.3	0.5	0.4	-	0.3	-	0.1	1.6	6.11 b	2.06 bc
	20 horas											
	CN	0.6	0,1	-	-	-	0.7	-	-	1,2	8.91 a	0.52 c
	CP	1.2	1.8	2.2	0.1	1	2	5.2	2.7	-	5.72 b	18.07 a
	FA	1.5	0.6	0.8	0.2	-	0.9	0.1	0.1	2.7	6.93 ab	3.88 b
	FB	1	0.3	1	0.3	-	1.1	-	0.1	2.3	5.56 b	3.85 b
	FC	1.5	0.3	0.4	0.3	-	0.2	-	-	0.3	5.57 b	1.98 bc
	72 horas											
	CN	0.6	0.1	-	-	-	0.7	-	-	1.2	8.91 a	0.52 b
	CP	1.2	1.8	2.2	0,1	1	2	5.2	2.7	-	5.72 b	18.07 a
	FA	0.7	0.1	0.1	0.4	-	-	-	-	0.6	6.53 ab	1.16 ab
	FB	2.1	0.1	0.3	0.4	-	0.1	0.1	-	1	4.97 b	1.98 ab
	FC	1.3	0.1	-	-	-	0.1	-	-	0.2	5.19 b	0.78 ab
chuvosa												
	contínuo											
	CN	-	-	-	-	-	-	-	-	-	9.75 a	0.0 b
	CP	0.3	1.2	0.7	0.2	0.2	0,4	1.1	1.7	-	4.97 b	10.95 a
	FA	0.8	0.4	0.4	-	-	0.2	-	-	1	10.09 a	0.82 ab
	FB	0.4	0.7	0.4	0.2	-	0.1	-	-	0.9	8.43 a	1.47 ab
	FC	1.6	0.5	0.5	-	-	-	-	-	1	8.39 a	1.10 ab
	20 horas											
	CN	-	-	-	-	-	-	-	-	-	9.75 a	0.0 b
	CP	0.3	1.2	0.7	0.2	0.2	0.4	1.1	1.7	-	4.97 c	10.95 a
	FA	0.9	0.3	-	-	-	-	-	-	0.3	7.30 ab	0.87 ab
	FB	2.9	0.1	-	-	-	-	-	0.2	0.3	4.66 b	0.96 ab
	FC	0.3	0.2	0.2	-	0.1	0.1	-	-	0.6	7.65 ab	0.68 ab
	72 horas											
	CN	-	-	-	-	-	-	-	-	-	9.75 a	0.0 c
	CP	0.3	1,2	0.7	0.2	0.2	0.4	1.1	1.7	-	4.97 c	10.95 a
	FA	0.5	0.4	0.3	-	-	-	-	-	0.7	7.53 ab	0.79 ab
	FB	1.6	1	0.1	0.4	0.1	0.4	-	0.1	1.6	4.53 b	11.81 a
	FC	0.3	0.5	0.2	-	-	-	-	-	0.7	6.11 b	2.21 cb

A- Micronúcleo; B- Anáfase com ponte; C- Anáfase multipolar; D- Atraso anafásico; E- quebra cromossômica; F- perda cromossômica; G- C-metáfase; H- aderência; I- célula binucleada; J- Índice mitótico; K- Freqüência de células aberrantes em relação as células em divisão. Médias seguidas pela mesma letra não diferem entre si no nível de significância de 5% pelo teste de Kuskal-Wallis feito para os postos médios.

unesp

Células irregulares observadas em raízes de *Allium cepa* tratadas com águas coletadas no rio Córrego dos Bagres em 2002.

pelodo	Tratamento	A	B	C	D	E	F	G	H	I	J	K
seca												
	contínuo											
	CN	2.2	1.6	-	-	-	-	-	-	-	11.36a	2.04b
	CP	4.4	2.2	0.8	0.9	0.5	0.5	1.2	3.9	0.1	6.81 bc	14.42 a
	FA	0.2	0.8	0.6	-	-	0.2	0.2	0.9	-	8.01 ab	1.88 c
	FB	1.4	0.1	0.2	0.2	-	1	1	2.4	0.6	6.59 c	5.72 ab
	FC						-				3.74 c	3.51 bc
	20 horas											
	CN	2.2	1.6	-	-	-	-	-	-	-	11.36a	2.04b
	CP	4.4	2.2	0.8	0.9	0.5	0.5	1.2	3.9	0.1	6.81 b	14.42 a
	FA	1	0.6	0.8	0.2	1.6	0.9	1	1.8	-	7.41 b	5.49 bc
	FB	0.2	2.2	1.3	0.2	0.6	1	2.3	2.3	-	7.11 b	7.58 b
	FC	0.2	0.7	0.9	-	0.2	0.8	0.9	3.2	-	7.32 b	4.86 bc
	72 horas											
	CN	2.2	1.6	-	-	-	-	-	-	-	11.36a	2.04b
	CP	4.4	2.2	0.8	0.9	0.5	0.5	1.2	3.9	0.1	6.81 b	14.42 a
	FA	2.2	0.9	0.2	-	0.9	1	1.6	1.8	0.2	7.43 ab	6.57 bc
	FB	0.6	-	0.2	-	0.4	0.2	2.8	5.9	0.1	3.64 c	11.93 abc
	FC	2.3	0.5	0.2	-	0.1	0.1	0.5	4.7	-	5.47 bc	7.62 bc
	recuperação											
	CN	2.2	1.6	-	-	-	-	-	-	-	11.36a	2.04b
	CP	4.4	2.2	0.8	0.9	0.5	0.5	1.2	3.9	0.1	6.81 a	14.42 a
	FA	1.0	0.3	1.0	-	-	0.6	0.6	-	0.3	1.77 a	3.35 b
	FB	1.0	1.0	0.4	1.0	1.4	5.2	6.8	2.20	0.2	5.05 a	23.67 a
	FC	1.9	-	-	0.75	1.0	0.82	1.15	1.45	0.4	5.15 a	9.78 a
chuvoso												
	contínuo											
	CN	2	-	-	-	0.8	0.1	0.1	-	-	8.29 a	0.18 c
	CP	1.8	1.6	2.0	-	1.8	0.8	4.4	1.0	-	1.43 b	15.81 a
	FA	0.3	0.7	0.6	-	1.1	0.5	0.4	0.3	0.1	8.37 a	5.07 b
	FB	-	0.7	0.2	0.1	0.1	0.2	0.6	0.2	-	5.99 a	3.79 a
	FC	0.2	0.8	0.4	0.1	0.1	0.4	1.3	1	1	6.68 a	7.69 ab
	20 horas											
	CN	2	-	-	-	0.8	0.1	0.1	-	-	8.29 a	0.18 a
	CP	1.8	1.6	2.0	-	1.8	0.8	4.4	1.0	-	1.43 b	15.81 b
	FA	0.6	0.5	0.6	-	0.4	0.5	0.9	0.5	0.3	8.04 a	5.22 a
	FB	0.4	0.6	0.4	0.1	0.2	0.2	1.7	1.5	0.1	7.24 a	7.36 a
	FC	0.5	0.5	0.5	0.1	0.6	0.5	0.7	1.1	0.3	6.95 a	6.76 a
	72 horas											
	CN	2	-	-	-	0.8	0.1	0.1	-	-	8.29 a	0.18 c
	CP	1.8	1.6	2.0	-	1.8	0.8	4.4	1.0	-	1.43 b	15.81 a
	FA	-	0.8	0.8	-	0.3	0.40	0.8	0.8	-	2.3 b	9.35 b
	FB	0.2	0.2	0.2	-	-	-	0.5	-	-	1.5 b	6.13 ab
	FC	0.3	0.6	1.20	-	1.40	1.0	3.0	1.0	-	2.8 b	13.27 b
	recuperação											
	CN	2	-	-	-	0.8	0.1	0.1	-	-	8.29 a	0.18 c
	CP	1.8	1.6	2.0	-	1.8	0.8	4.4	1.0	-	1.43 c	15.81 a
	FA	1.2	0.5	0.2	-	0.4	0.4	0.3	0.7	-	6.13 ab	5.81 b
	FB	0.9	0.6	-	-	0.6	0.1	0.5	0.4	-	4.48 b	6.76 b
	FC	0.6	0.6	-	0.1	0.3	0.1	0.3	0.7	-	3.93 b	6.60 b

A- Micronúcleo; B- Anáfase com ponte; C- Anáfase multipolar; D- Atraso anafásico; E- quebra cromossômica; F- perda cromossômica; G- C-metáfase; H- aderência; I- célula binucleada; J- Índice mitótico; K- Freqüência de células aberrantes em relação as células em divisão. Médias seguidas pela mesma letra não diferem entre si no nível de significância de 5% pelo teste de Kuskal-Wallis feito para os postos médios.

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