PROTECTIVE EFFECT OF PROBIOTIC BACTERIA AGAINST CADMIUM-INDUCED GENOTOXICITY IN RAT HEPATOCYTES IN VIVO AND IN VITRO

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Abstract - The protective effect of probiotic bacteria against cadmium (Cd)-induced genotoxicity was studied in rat hepatocytes in vivo and in vitro. Male Wistar rats, Rattus norvegicus, were treated for five weeks with (i) CdCl₂ (70 ppm in the drinking water), (ii) a mixture of lyophilized probiotic bacteria Lactobacillus rhamnosus, L. acidophilus and Bifidobacterium longum (5×10⁸ cfu/g of food), or (iii) CdCl₂ and probiotic bacteria. In addition, single cells obtained from the untreated rat liver were exposed to CdCl₂ (70 ppm), probiotic bacteria (1.28 mg/ml), or CdCl₂ and probiotic bacteria, for 15 min at 22°C in the dark. The level of Cd-induced DNA damage in hepatocytes was determined by the comet assay. The obtained results show that probiotic bacteria significantly reduced Cd-induced genotoxicity, both in vivo and in vitro (20% and 48%, respectively). Moreover, the toxicity of Cd to lactobacilli in the gastrointestinal tracts of rats was significantly decreased in the probiotic-treated animals. The binding of Cd²⁺ to probiotic bacteria was proposed as the most probable protection mechanism.

Key words: Cadmium, toxicity, lactic acid bacteria, genotoxicity, rat hepatocytes

INTRODUCTION

Cadmium (Cd) is a non-essential heavy element that represents a serious environmental hazard (Satarug et al., 2003). Human exposure to Cd occurs through food, air, water, tobacco smoke, industrial products and by occupational exposure (Fowler, 2009). Cd is extremely harmful for human beings and animals (Jarup, 2003; Waalkes, 2003). It has been found to be toxic to almost every organ in the body, causing hypertension, renal tubular dysfunction, bone fracture and cancer (Jin et al., 2003; Satarug et al., 2003). Cd has been classified as a group I carcinogen by the International Agency for Research on Cancer (Boffetta, 1993), and as a group B1 (probable human carcinogen) by the Environmental Protection Agency (Fotakis and Timbrell, 2006).

The mechanisms by which Cd induces a wide variety of adverse health effects and cancer are poorly understood, but induction of reactive oxygen species and inhibition of DNA repair seem to have a predominant role at the molecular level (Filipic et al., 2006; Rao, 2009). Prolonged exposure to low concentrations of Cd can increase glutathione levels, whereas high Cd concentrations lead to glutathione depletion in vitro. In vivo and in vitro depletion of glutathione results in increased toxicity following the administration of Cd (Fotakis and Timbrell, 2006). In addition to oxidative stress, inflammation has also been implicated in Cd-induced tissue injury (Oteiza
et al., 1999). Several antioxidants and anti-inflammatory agents were found to be effective in minimizing Cd-induced organ damage (Sen Gupta et al., 2004; Kara et al., 2007; Yadav and Khandelwal, 2008; Abdel-Aziem et al., 2011).

Cd has long been considered a non-genotoxic carcinogen, and the evidence that Cd²⁺ causes gene mutations in bacteria or mammalian cells was weak. However, alterations in testing protocols have recently led to better evidence of its mutagenicity, especially in bacteria. The fact that bacterial and mammalian cells appear to sustain some type of repairable DNA damage after exposure to Cd suggests that the damage is caused in an indirect manner. Cd-metallothionein complex is able to cause DNA strand breaks and to induce a “pro-oxidant state” by causing a depletion of cellular glutathione. It has also been suggested that the mechanism responsible for the genotoxicity of Cd may involve the direct interaction with DNA through the binding of Cd²⁺ at G, A and T bases (Valverde et al., 2001; Hossain and Huq, 2002). Cd can also inhibit DNA repair, and can therefore act synergistically with certain mutagens and carcinogens (Rossman et al., 1992). The inhibition of an essential DNA mismatch repair by Cd compounds results in a high level of genetic instability (Jin et al., 2003).

Numerous investigations indicate that probiotic bacteria can play an important role in the body’s natural processes of detoxification and elimination of Cd (Haskard et al., 2001; Rao, 2009). Their beneficial properties are related to their capacity to adhere to or bind different targets. Probiotic bacteria such as Lactobacillus rhamnosus GG strain (ATCC 53103) and L. rhamnosus LC-705 strain (DSM 7061) can bind toxic compounds, such as aflatoxin B1 (Haskard et al., 2001), mutagens from food (Turbic et al., 2002), or microcystin-LR (Halltunen et al., 2007).

In this work, we have assessed the effect of commercial preparations of probiotic bacteria, Lactobacillus rhamnosus, L. acidophilus and Bifidobacterium longum on Cd-induced genotoxicity in rat hepatocytes. Male Wistar rats were treated with CdCl₂, a lyophilized mixture of probiotic bacteria, or CdCl₂ and probiotic bacteria for five weeks. In addition, single cells obtained from the livers of untreated rats were exposed to CdCl₂, probiotic bacteria, or CdCl₂ and probiotic bacteria in vitro. The level of Cd-induced DNA damage in the hepatocytes was determined by comet assay. In addition, we evaluated the number of lactobacilli and bifidobacteria in the feces of treated animals in order to assess the effect of the probiotic on Cd-induced toxicity to bacterial microflora in rat gastrointestinal tract.

MATERIALS AND METHODS

Animals

Male Wistar rats (Rattus norvegicus), weighing 130±10 g, were acclimatized to 22±2°C in metabolic cages (3 rats/cage) and maintained under a 12 h light/dark cycle. The rats were fed with commercial rat food and drank tap water ad libitum.

Probiotic bacteria

The probiotic used in this study was the commercial preparation PROBIOTIC®, Ivančić i sinovi d.o.o., Belgrade, Serbia. The capsules are declared to contain 5x10⁹ lyophilized cells of Lactobacillus rhamnosus Rosell-11, Lactobacillus acidophilus Rosell-52 and Bifidobacterium longum Rosell-175 strains.

Chemicals, media, and culture conditions

Cadmium chloride (CdCl₂ analytical grade) was from Fisher Scientific UK. Hank’s BBS (HBSS, Lot No. H00911-0648) and Dulbecco’s PBS (Lot No. H00911-1191) were from PAA Laboratories GmbH, Austria. Lactobacilli were cultured on MRS agar (ScharlauChemie S.A., Spain) for 48 h at 37°C. Bifidobacteria were plated on Bifidobacterium agar (HiMedia, India) in anaerobic conditions for 24 h at 37°C. Peptone water “Torlak”, Belgrade (Lot. No. 364201) contained 10 g pepton-4 “Torlak”, 5 g NaCl, 0.01 g Fuchsin S per liter of distilled water. L. rhamnosus, L. acidophilus and B. longum were identified.
using an API 50 CH identification kit (bioMerieux, Lot. No. 842019201).

**In vivo exposure**

The rats were randomly divided into four groups of 6 males each. All groups were treated for five weeks as follows: Group 1 (control group) was given food and tap water *ad libitum*; Group 2 was given the probiotic mixed with food at a dose of 5×10⁸ colony forming units (cfu)/g of food; Group 3 received CdCl₂ at a dose of 70 ppm in the drinking water, and Group 4 received both CdCl₂ and probiotic. After sacrificing the animals, the livers were processed to obtain single-cell suspensions and the comet test was performed to assess DNA damage.

**Preparation of single-cell suspensions**

Single-cell suspensions of liver tissue were prepared using a method adapted from Wilson et al. (1998). The liver was excised and chopped separately 10 times in 0.2 ml of HBSS using two fresh scalpel blades in a scissor-like movement on a Petri dish, washed off gently into a 15 ml centrifuge tube with a further 2.8 ml HBSS and 0.03 ml of 0.5% trypsin. The suspension was gently rocked for 10 min at room temperature, after which 10 ml of HBSS was added and the suspension passed through a 40 µm sieve to remove any large fragments that remained. After centrifugation (800 g for 5 min), the supernatant was discarded and the pellet carefully re-suspended in 1 ml of HBSS. Cell viability was measured by trypan blue dye exclusion method (Altman et al., 1993) and cell density was adjusted to 3 × 10⁵ cell/ml.

**In vitro exposure**

The single-cell suspension obtained from the untreated rat liver (control group) was used to prepare comet slides embedded in agarose as described below. The slides were exposed to CdCl₂ (70 ppm), probiotic (1.28 mg/ml) and a combination of CdCl₂ and probiotic for 15 min at 22°C in the dark. Slides exposed to PBS only served as controls. The concentrations of Cd and probiotic bacteria corresponded to the concentrations for *in vivo* treatment. After exposure, the slides were rinsed with PBS to remove residues and submitted to comet assay procedure as described below.

**Comet assay**

The assay was performed as described by Tice et al. (2000). The cell suspension (30 µl) was mixed with 70 µl of 1% LMP (low melting point) agarose and added to slides, previously precoated in 1% NMP (normal melting point) agarose. The slides were incubated in lysis buffer (2.5 M NaOH, 0.1 M EDTA, 0.01 M Tris and TritonX-100, pH 10) for 1 h at 4°C, transferred into an electrophoresis solution (300 mM NaOH, 1 mM EDTA, pH 13) for 20 min to allow DNA unwinding, and subjected to electrophoresis for 20 min at 25 V and 300 mA. Finally, the slides were neutralized with 0.4 M Tris buffer (pH 7.5), stained with 20 µl ethidium bromide (5 µg/ml), and analyzed using a fluorescence microscope (Leica) and image analysis software (Comet IV, Perceptive Instruments). Fifty nuclei were analyzed per experimental point (in triplicate), and the percentage of the fluorescence in the comet tail was scored as a reflection of DNA damage.

**Identification of probiotic bacteria**

To obtain pure cultures of probiotic bacteria, MRS broth was inoculated with the content of one capsule of PROBIOTIC® and incubated at 30°C. After 48 h the culture was streak-inoculated on MRS agar and *Bifidobacterium* agar and incubated for 48 h at 30°C aerobically and for 24 h at 37°C anaerobically, respectively. Using Gram staining and an API 50 CH identification kit we identified small grey colonies on the MRS agar as *Lactobacillus rhamnosus*, big white colonies on the same medium as *Lactobacillus acidophilus* and colonies on the Bifidobacterium agar as *Bifidobacterium longum*.

**Microbiological analysis of feces**

Feces samples from the four treatment groups were
collected and immediately transported to the laboratory. A feces specimen (4 g from each group), was suspended in 36 ml of peptone water and homogenized. Samples were vigorously vortexed for 1 min and then centrifuged at 65 g for 5 min to deposit any remaining solid matter. The number of lactobacilli and bifidobacteria was determined by spreading appropriate dilutions onto the MRS agar and Bifidobacterium agar plates, respectively. Small grey colonies on the MRS were considered to be *Lactobacillus rhamnosus*, while big white colonies on the same medium were considered to be *Lactobacillus acidophilus*. Experiments were performed twice, each with duplicate samples.

**Statistical analysis**

Data for bacterial enumeration were statistically evaluated by one-way analysis of variance (ANOVA), using SigmaStat (Version 3.1) software. Holm-Sidak comparisons were performed when ANOVA was significant. The level of significance was set at p<0.05. For the results of the comet assay, one-way analysis of variance (non-parametric ANOVA, Kruskal-Wallis test) was used to analyze differences between the treatments within each experiment. The Duncan Post Hoc test was used to compare median values of the percent of fluorescence in comet tails for all treatments; p<0.05 was considered as statistically significant.

**RESULTS**

**The effect of probiotic on Cd-induced genotoxicity in rat hepatocytes**

Two experimental procedures, *in vivo* and *in vitro*, were used to study and compare the effect of a probiotic on Cd-induced genotoxicity in rat hepatocytes. As shown in Fig. 1, the percent of DNA damage in control and probiotic-treated cells was similar in both experimental conditions. The percent of DNA in the comet tail was 1.71% in the controls, 1.53% *in vivo* and 1.64% *in vitro* after the treatment with probiotic. The treatment with Cd resulted in a significant increase in DNA damage: the percent of DNA in the comet tail increased from 1.71% to 8.46% *in vivo* and from 1.71% to 3.43% *in vitro*. When the animals or cells were simultaneously treated with Cd and probiotic there was a significant decrease of Cd-induced genotoxicity. The percent of DNA in the comet tail declined from 8.45% to 6.78% *in vivo* (a 20% decrease) and from 3.43% to 1.82% *in vitro* (a 48% decrease).

The number of lactic acid bacteria in the feces of rats exposed to Cd and probiotic

The number of bifidobacteria in the feces of rats from all groups is shown in Fig. 2. While there was no significant difference between the control and probiotic-treated groups (1.0 x 10⁷ and 1.1 x 10⁷ cells/g of feces), exposure to Cd significantly decreased the abundance of bifidobacteria compared with control group (by 64%). Adding probiotic to Cd resulted in an increase of bifidobacteria counts, from 3.6 x 10⁶ to 4.6 x 10⁶ cells/g, but this difference was not statistically significant.

The number of *L. acidophilus* and *L. rhamnosus* in the feces of differently treated rats is shown in Figs. 3 and 4. In the probiotic-treated animals, the number of *L. acidophilus* was significantly higher (1.2 x 10⁸ cells/g) compared with the control (9.3 x 10⁷ cells/g). Exposure to Cd significantly reduced *L. acidophilus* counts compared with the control (by about 95%). In animals simultaneously treated with Cd and probiotic the number of *L. acidophilus* was significantly increased, from 4.5 x 10⁶ to 5.5 x 10⁷ cells/g of feces.

As in the case of *L. acidophilus*, the number of *L. rhamnosus* in probiotic-treated animals was significantly higher than in the control (9.2 x 10⁵ and 5.5 x 10⁵ cells/g, respectively). Exposure to Cd resulted in the significant reduction of *L. rhamnosus* counts compared with the control (by 40%). Interestingly, treatment with the probiotic completely diminished Cd-induced toxicity. The number of *L. rhamnosus* increased from 3.3 x 10⁵ to 1 x 10⁶ cells/g of feces, which is insignificantly different from the number in the probiotic-treated animals (9.2 x 10⁵ and, respectively).
**Fig. 1.** The effect of Cd and probiotic on DNA damage in rat hepatocytes exposed in vivo and in vitro. The level of DNA strand breaks is expressed as the percentage of DNA in the comet tails. Fifty cells were analyzed per experimental point. *significantly different from the control group (p<0.05)*

**Fig. 2.** The number of fecal bifidobacteria in Cd-exposed rats, with or without probiotic. Presented values are the means of two independent experiments ± SD. *above the bar indicates significant difference from control group; *inside the bar indicates significant difference from probiotic group (p<0.05)
Fig. 3. The number of fecal *L. acidophilus* in Cd-exposed rats with or without probiotic. Presented values are the means of two independent experiments ± SD. *above the bar indicates significant difference from control group; *inside the bar indicates significant difference from probiotic group (p<0.05)

Fig. 4. The number of fecal *L. rhamnosus* of in Cd-exposed rats with or without probiotic. Presented values are the means of two independent experiments ± SD. *above the bar indicates significant difference from control group; *inside the bar indicates significant difference from probiotic group (p<0.05)
DISCUSSION

A number of reports have convincingly demonstrated the genotoxic potential of Cd in animals and mammalian cells (Waisberg et al., 2003; Bertin and Averbeck, 2006). In the present work, we investigated the protective effect of probiotic bacteria against liver genotoxicity induced by Cd, using the comet assay. The experiments were performed in vivo and in vitro, and the genotoxic effect of Cd was significant in both cases. From our data it is obvious that after chronic in vivo exposure to Cd (5 weeks) the percent of DNA damage was higher compared to short in vitro exposure (15 min). It is known that the genotoxicity of Cd involves the induction of oxidative stress and inhibition of different DNA repair mechanisms (Rossman et al., 1992; Jin et al., 2003; Rao, 2009; Jomova and Valko, 2011). It is to be expected that both processes occurred during the chronic in vivo exposure, leading to substantial DNA damage. In the case of in vitro exposure, the time was probably too short for saturation of the antioxidative defense of the hepatocytes, and the amount of DNA lesions was consequently lower. In addition, the low concentration of vitamin C present in the PROBIOTIC® preparation could have acted as an antioxidant and reduced oxidative stress (Sen Gupta et al., 2004).

Nowadays there is an increasing interest in the use of probiotic bacteria to mitigate the toxic/genotoxic potential of heavy metals. On the basis of our results it is clear that the protective effect of probiotic bacteria against Cd-induced genotoxicity exists. The protection was more pronounced in in vitro conditions (48% compared to 20% in vivo), indicating that the most probable mechanism was the direct binding of Cd$^{2+}$ to the probiotic bacteria. The surface of lactic acid bacteria is composed of a thick layer of peptidoglycan, teichoic acid, proteins and polysaccharides; moreover, some Bifidobacterium strains and L. rhamnosus GG are known to produce exopolysaccharides. These structures contain different kinds of negatively charged groups such as carboxyl, hydroxyl and phosphate groups. Therefore, probiotic bacteria have a great number of different possible ligands able to bind cations such as Cd$^{2+}$ (Teemu et al., 2008). It is possible that in the mixture of liver cells, probiotic bacteria and Cd in vitro, bacterial cells bind Cd$^{2+}$ more efficiently than in the rat intestines, preventing more effectively the induction of DNA damage. The ability of probiotic strains to perform auto-aggregation, as well as co-aggregation with other bacteria in the intestines, might reduce the binding of heavy metals (Kos et al., 2003; Salim Abdelgader et al., 2011), which could be one of the reasons for the weaker protection observed in vivo.

Heavy metals have inhibitory effects on the growth of a number of bacteria (Forsberg, 1978; Ravikumar et al., 2007). In this study, we noticed significant reduction in the counts of all the studied bacteria in the feces of rats treated with Cd compared with the control group. The toxicity increased, in order L. rhamnosus (40%), bifidobacteria (64%), and L. acidophilus (95%), indicating the highest resistance of L. rhamnosus to Cd. Our results are similar to those of Fazeli et al. (2011), who reported that Cd has a toxic effect on gastrointestinal bacteria in mice. The cytotoxicity of Cd is well documented, and it appears that it is mainly due to the oxidative deterioration of biomolecules, including DNA, proteins and lipids (Bertin and Averbeck, 2006).

The natural inhabitants of the intestine play a pivotal role through different mechanisms in the physiological functions of the organism and pathological processes. Knowledge is accumulating that lactic acid bacteria modulate gut physiology, immunological functions, and may produce many beneficial effects (Erickson and Hubbard, 2000). This has led scientists to investigate the efficacy of probiotics, prebiotics and synbiotics in the prevention and treatment of diseases and toxicities. Administration of probiotics enhances the intestine immune system, decreases bacteria translocation and prevents the overgrowth of enteric pathogens; it also has beneficial effects on acute hepatocellular disease (Fernandes et al., 1987; Mao et al., 1996; Adawi et al., 2001; Salminen et al., 2010). The mechanisms by which probiotics exert biological effects are still poorly understood. The adsorption of heavy metals to the bacterial surface appears to be the main mechanism for their removal by
probiotics. Halttunen et al. (2008) found that different lactic acid bacteria were effective in the removal of heavy metals, microcystin-LR and aflatoxin B1. Moreover, they found clear differences in metal and toxin removal efficiency between the strains. The assessment of Cd\(^{2+}\) removal also indicated a strongly pH-dependent process, with the highest binding at a neutral pH (Halttunen et al., 2007). Knowing that the pH values in different parts of the gastrointestinal tract of rats vary from 5.3-7.4 (McConnell et al., 2008), we can propose that the highest binding of Cd\(^{2+}\) occurs in the large intestine where pH values are close to neutral.

Rowland et al. (1998) reported that feeding rats with Bifidobacterium longum decreased the levels of ammonia in the caecum and reduced the incidence of preneoplastic lesions induced by azoxymethane. On the contrary, our results do not indicate a protective effect of bifidobacteria against Cd-induced toxicity. In the group of rats treated with probiotic, the number of L. acidophilus and L. rhamnosus in the feces significantly increased in comparison with the control, while the number of bifidobacteria remained unchanged. Moreover, in the group treated with Cd plus probiotic, the number of L. acidophilus and L. rhamnosus significantly increased compared to the Cd-treated group, while the increase of bifidobacteria counts was insignificant. Obtained results point at the different behavior of bifidobacteria and lactobacilli in relation to binding Cd\(^{2+}\). It seems that lactobacilli possess some capacity to bind Cd\(^{2+}\), which enables their better survival. However, the probiotic potential of different bacterial strains, even within the same species, differs. Different strains of the same species may have different areas of adherence (site-specific), specific immunological effects, and the actions on healthy/inflamed mucosa may be distinct from each other (Soccol et al., 2010).

CONCLUSIONS

This study reports on the efficiency of probiotic bacteria against Cd-induced toxicity to gut microflora and genotoxicity to liver cells. The health-promoting properties of probiotic bacteria and the implications from the present study indicate that the application of probiotics may be a good approach for counteracting the negative effect of Cd and possibly other heavy metals. Further research is needed to define a specific probiotic combination that would be the most effective in binding Cd\(^{2+}\) and other heavy metals, as well as different toxins and carcinogens.

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REFERENCES


