Comet assay studies indicate that caffeine-mediated increase in radiation risk of embryos is due to inhibition of DNA repair

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It is well known that under specific conditions caffeine is able to enhance radiation risk of mammalian cells by a factor of ~1.5–2. Various mechanisms are discussed in the literature as possible explanations for this interaction. Inhibition of DNA repair plays a crucial role in the discussion, although direct evidence for this assumption is difficult to obtain. We used the ‘comet assay’ in order to analyse the significance of repair inhibition by caffeine in the two-cell stage of mammalian gestation. Our data show that at the concentration necessary for increasing radiation risk (2 mM), caffeine effectively inhibits the restitution of radiation-damaged DNA.

Introduction

Caffeine is one of the most widespread stimulants in the world (as a constituent of coffee, tea, maté and cola beverages). Numerous studies have shown that under specific conditions caffeine is able to increase radiation risk (for reviews see Kihlman, 1977, pp. 363–406; Streffer and Müller, 1984, pp. 187–190). Most of these conditions can be met only by using rather artificial experimental designs, e.g. extraordinarily high caffeine concentrations (~1–2 mM, corresponding to several hundred cups of coffee or tea per day). At low concentrations, no effect on radiation risk is observed, because either the experimental systems are too insensitive or no such effect occurs. In order to distinguish between these two possibilities, the knowledge of the mechanism by which caffeine acts is urgently required.

Some years ago, we observed that, at high concentrations, caffeine potentiates the radiation effect on preimplantation mouse embryos (Müller et al., 1983, 1985, 1993; Müller and Spindle, 1986; Müller, 1989). Preimplantation embryos were chosen for several reasons: (i) the locomotion of the four-cell stage, embryos develop in a synchronous way, i.e. at a given time all the cells are in the same cell cycle phase (this is an important aspect, because radiation sensitivity changes according to the distribution within the cell cycle); (ii) embryos are very sensitive to many agents; (iii) embryos represent complete organisms (in contrast to cells of a conventional cell culture); and (iv) embryos are crucial for the maintenance of the species.

An isobologram analysis (for an explanation see Streffer and Müller, 1984) of the embryonic response showed that the observed increase in radiation risk in the presence of caffeine was due to an interaction between the mechanisms occurring after exposure to ionizing radiation and caffeine. A favorite candidate for the mode of interaction was inhibition of DNA repair exerted by caffeine.

As the experiments were done with two-cell embryos, only few cells were available for the analysis of the mechanism responsible for the increase in risk after combined exposure to caffeine and radiation. Unfortunately, no method existed at that time to study repair in such small numbers of cells. This has changed since the introduction of the ‘comet assay’ by Östling and Johanson (1984). In this assay cell nuclei are exposed to a weak electric field. Dependent on the extent of nuclear damage after, for example, radiation exposure, ‘comets’ are formed, in which the DNA amount in the ‘comet tail’ is a measure of DNA damage. If the cells are incubated at optimum conditions for some time, the time-dependent decrease in the size of the comet tail is a measure for the repair capacity of these cells. [For reviews of the comet assay see McKelvey-Martin et al. (1993) and Fairbairn et al. (1995).] Although the Östling–Johanson technique is carried out at almost ‘neutral’ conditions (lysis buffer pH = 9.5), the major type of DNA damage determined is not the double- but the single-strand break (perhaps together with strand break independent types of DNA damage). This is primarily due to the rather weak lysis conditions (15 min at room temperature), which result in only a partial removal of proteins. Thus, it seems to be the effect of single-strand breaks on DNA supercoiling (reminiscent of the ‘halo’ assay) that is determined under these conditions (Fairbairn et al. 1995). These considerations are well in line with the comparatively high sensitivity of the method under our conditions [significant differences to the unirradiated control at 0.1 Gy (Müller et al., 1994)]. Also, increasing the lysis pH to 11.5 under otherwise identical conditions increased the sensitivity of the assay only marginally (unpublished results).

The possibility of determining DNA damage and DNA repair in a few individual cells gave us the opportunity to test the hypothesis of repair inhibition by caffeine in preimplantation embryos. It turned out that indeed a marked inhibition of the speed and completeness of the restoration of DNA after radiation damage was exerted by the presence of caffeine, i.e. caffeine inhibited the repair of radiation-induced DNA damage.

Materials and methods

Embryo collection and culture

Detailed descriptions of embryo collection and culture have already been published (e.g. Müller, 1989). In the following only a brief outline is given. In order to obtain a synchronously growing population of embryos, three non-supervoluted females of the HLG/Zoe strain were mated with one male from 7 to 9 a.m.; subsequently the females were checked for a vaginal plug, indicating copulation. Isolation of embryos was carried out by flushing excised oviducts with Brinster’s medium for ovum culture [BMOC-2 (Brinster, 1969)] at 26 h post-conception. Embryos were collected in a 100 μl drop of BMOC-2 at 37°C in a humidified atmosphere with 10% CO2. After isolation, the embryos were left in the incubator until radiation exposure.

Animal experiments were carried out according to the current version of the German Law on the Protection of Animals.

Zona pellucida removal

The zona pellucida had to be removed prior to the comet assay in order to guarantee some displacement of the blastomeres and of the polar bodies to
avoid overlapping of comets. As the experiments for detecting initial DNA damage were done at 0°C, it was not possible to remove the zona pellucida by an enzymatic reaction (e.g. by pronase (Marticorena et al., 1983)).

Therefore, we checked two other approaches for their applicability.

Heat denaturation. Glass slides were precoated with 0.1% agarose and subsequently covered with 1 ml 0.75% agarose at a temperature >70°C. Immediately thereafter, the intact two-cell embryos were placed in a marked area of the hot gel and left on a hotplate (50°C) for 5 min. This procedure resulted in the breakdown of the zona pellucida and the separation of the blastomeres and polar bodies. For gelling, the slides were put on a cooling plate (0°C) for 5-10 min.

Acid denaturation. Embryos were suspended in a 200 μl drop of acidic PBS (phosphate-buffered saline, pH 2.5) at room temperature. Great care was taken to keep the embryos in suspension and to avoid contact of the embryos with the bottom of the Petri dish, because under these conditions embryos become extremely adhesive. As soon as the zona pellucida dissolved (after ~15 s), the embryos were removed from acidic PBS, washed briefly with BMOC-2 and transferred into agarose (500 μl; 0.75%).

Since only a limited number of embryos was available per experiment, it was sometimes difficult to relocate the comets under the microscope. For that reason, we tried to carry out the electrophoresis with blastomeres suspended in a 50 μl droplet of agarose placed in the middle of a precoated slide. This technique, however, resulted in small comets, mostly probably due to an insufficient electric field within the agarose droplet. In all the experiments reported in this paper, therefore, blastomeres were suspended in 500 μl of agarose spread over the entire slide. Each slide carried a circle scratched with a diamond pen on the reverse side, marking the approximate position of the blastomeres.

Radiation exposure

All irradiations were carried out 30 h post-conception using a Stabilipan X-ray machine (Siemens) with a 0.5 mm copper filter at 240 kVp and 15 mA. In those experiments in which the initial DNA damage following radiation exposure was determined, the embryos were placed on ice before and after irradiation to avoid effects by repair processes; immediately after radiation exposure the comet assay was carried out. In the repair experiments, embryos were exposed to X-rays at room temperature and subsequently transferred back to the incubator. The comet assay was carried out after the corresponding repair times (15, 30, 60 and 120 min) at 37°C.

Comet assay

In the case of acidic removal of the zona pellucida, blastomeres which did not detach from each other spontaneously were separated by pulling them up and down a pipette; subsequently, the blastomeres were embedded in 500 μl of warm (43°C) 0.75% agarose spread homogeneously on a precoated (0.1% agarose) microscopic slide. The procedure of heat removal of the zona pellucida has been described above. The slides were then cooled down to 0°C on a cooling device.

The comet assay was carried out essentially as described by Müller et al. (1994). Slight modifications had to be introduced: slides were submersed in a lysis solution of 2.5% sodium dodecyl sulphate + 0.025 M EDTA + 1% N-lauroylsarcosine (Sigma), pH 9.5, for 15 min. Following a short washing step (5 min in distilled water) the electrophoresis was carried out in TBE buffer (117 mM Tris, 91 mM borate, 3 mM EDTA, pH 8.4) on a flatbed apparatus (FBE-3000, Pharmacia) at 2.5 V/cm for 5 min at 10°C. After washing in distilled water for 5 min and air-drying on a hotplate (40°C) for 30 min, the slides were stored in a humidified container at 4°C. This procedure increases the time for which the slides can be stored without any loss of quality to ~2 weeks and prevents contamination, in particular by moulds; storing them without previous drying results in a rather rapid deterioration of the comets.

Before staining, the gels were rehydrated in distilled water for 15 min. Each slide was stained with 2 μg propidium iodide (2.5 X 10⁻⁴ M) and incubated in a humidified container until quantitative DNA determination. The fluorescence signal was detected using a Leitz MPV2 fluorescence microscope (excitation at 530-560 nm, detection >580 nm) and a CCD camera with image intensifier connected to a self-designed image analysis system. Measurements were done at 250X magnification, analysing the total fluorescence of each comet and the fluorescence of the head of the comet. The tail/head ratio of the DNA content was calculated as a measure of DNA damage.

Results

Figure 1 reveals a linear increase in the amount of DNA found in the comet tails up to 2 Gy. Higher doses, however, are somewhat less efficient in producing comet tails, so that a comparatively smaller additional increase is seen after 5 Gy (the tail/head ratio increases by a factor of 4.3 between 2 Gy and controls, whereas the increase from 2 to 5 Gy amounts to only a factor of 1.5). Thus, the shape of the dose-response curves is somewhat different to those observed for other cell lines in our institute (tumour cells, lymphocytes, fibroblasts), which show a linear component only at doses of <1 Gy. Figure 1 also reveals that the mode of removal of the zona pellucida affects the amount of DNA damage considerably. The most gentle treatment was by acidic PBS (pH 2.5), whereas heat (>70°C) induced significantly more DNA damage. For this reason, we used acidic PBS for removal of the zona in the repair experiments. We also reduced the amount of agarose (from 1 to 0.5 ml) in order to improve the recovery of the embryos. The comet formation was not affected by this reduction.

The repair results are summarized in Figure 2. Looking at the repair kinetics after radiation exposure alone (2 Gy) shows that early embryos are able to repair DNA damage, as measured by the comet assay, rather rapidly. Two-thirds of the damage had disappeared after 15 min and control levels were reached after 1 h. This is clearly faster than in tumour cell lines (Muller et al., 1994), fibroblasts and lymphocytes (unpublished results obtained by using identical assay conditions).

A concentration of 2 mM caffeine does not increase the extent of initial DNA damage; an exposure to 5 mM caffeine for 120 min is required for a measurable increase in the amount of DNA found in the comet tail (data not shown). Repair of radiation-induced damage is, however, inhibited markedly in the presence of 2 mM caffeine (Figure 2). All parts of the curve seem to be affected: the initial processes occur at a lower speed and after 2 h the residual damage is still >40% of the original damage measured by the comet assay. Thus, caffeine effectively diminishes repair of radiation-induced DNA damage.

Discussion

In previous experiments we found that high concentrations of caffeine are able to increase radiation risk of early embryonic...
However, are well known. In the following, the most prominent replication repair predominate. Exceptions to these rules, Timson, 1977. Common to almost all of these mechanisms is most frequently, whereas in mammalian cells effects on post-
radiation and caffeine have been suggested (for reviews see Kihlman era/., 1974; Kihlman, 1977, in particular pp. 402^05;

Enhances radiation risk, in some cases it just adds its effects to the radiation effects and in some cases it even diminishes
understanding, how-
We used the comet assay because of its ability to analyze DNA damage and repair in very few, individual cells. The assay had to be adapted to the peculiarities of the experimental system. Thus, the gel volume had to be reduced in order to facilitate the re-detection of the embryonic nuclei on the slides and to improve the staining of the nuclear material without affecting the tail/head ratio of the comets.

In the experiments described in this paper, exactly the same conditions have been used as in our previous studies (Müller et al., 1983, 1985). Repair was allowed for only 2 h to avoid complications by embryos dividing into the four-cell stage (radiation exposure took place late in the G2 phase of the cell cycle, a situation comparable to the previous experiments). This problem is most serious in the combined group (X-rays + caffeine), because caffeine reduces the radiation-induced G2 block markedly (see above).

In our experiments, caffeine at a concentration that did not induce DNA damage affected repair processes in two respects: firstly, the speed of repair was reduced, and secondly, the residual damage after 2 h was considerably higher than in the embryos exposed to radiation only. A third aspect is worth mentioning: it has frequently been reported that caffeine might affect specific repair enzymes directly (for a review see Kihlman, 1977, pp. 339–354). Two mechanisms were identified by Selby and Sancar (1990a, 1990b) in Escherichia coli explaining the mode of inactivation at least for excision repair enzymes.

In order to decide whether inhibition of repair by caffeine makes a contribution to the previously observed increase in radiation risk in early embryonic stages, we determined the speed and efficiency of DNA restoration after radiation exposure of two-cell embryos in the absence and presence of caffeine. We used the comet assay because of its ability to analyze DNA damage and repair in very few, individual cells. The assay had to be adapted to the peculiarities of the experimental system. Thus, the gel volume had to be reduced in order to facilitate the re-detection of the embryonic nuclei on the slides and to improve the staining of the nuclear material without affecting the tail/head ratio of the comets.

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This paper has contributed to solving the problem of interaction between radiation and caffeine by showing that at least part of the increased radiation risk in the presence of caffeine

![Graph](https://example.com/graph.png)
comes from repair inhibition via mechanisms of the types (i), (ii) and/or (vi) above. This does not mean that the other mechanisms are ruled out. For example, it is reasonable to assume that the reduction of the radiation-induced G₂ block augments the problems for the cells, because in addition to repair inhibition the period available for repair processes is diminished.

Eventually, it may turn out that all of the mechanisms mentioned above are involved in the interaction between radiation and caffeine and that only the extent of the contribution of a specific mechanism varies from cell type to cell type.

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References


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