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Low concentrations of caffeine induce asymmetric cell division as observed in vitro by means of the CBMN-assay and iFISH

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ABSTRACT

The dual role of caffeine as a chromosomal damage inducer and G2/M-checkpoint abrogator is well known but it is observed mainly at relatively high concentrations. At low concentrations, caffeine enhances the cytogenetic effects of several carcinogens and its intake during pregnancy has been recently reported to cause adverse birth outcomes. Interestingly, a threshold below which this association is not apparent was not identified. Since chromosomal abnormalities and aneuploidy are the major genetic etiologies of spontaneous abortions and adverse birth outcomes, we re-evaluate here the effects of caffeine at the cytogenetic level and propose a model for the mechanisms involved. Our hypothesis is that low caffeine concentrations affect DNA replication and cause chromosomal aberrations and asymmetric cell divisions not easily detected at metaphase since damaged cells are delayed during their G2/M-phase transition and the low caffeine concentrations cannot abrogate the G2-checkpoint. To test this hypothesis, caffeine-induced chromatid breaks and micronuclei in peripheral blood lymphocytes (PBLs) were evaluated in vitro after low caffeine concentration exposures, followed by a short treatment with 4 mM of caffeine to abrogate the G2-checkpoint. The results show a statistically significant increase in chromatid breaks at caffeine concentrations ≥1 mM. When caffeine was applied for G2/M-checkpoint abrogation, a statistically significant increase in chromatid breaks, compared to an active checkpoint, was only observed at 4 mM of caffeine. The potential of low concentrations to induce asymmetric cell divisions was tested by applying a methodology combining the cytochalasin-B mediated cytokinesis-block micronucleus assay (CBMN) with interphase FISH (iFISH), using selected centromeric probes. Interestingly, low caffeine concentrations induce a dose dependent aneuploidy through asymmetric cell divisions, which are caused by misalignment of chromosomes through a mechanism unrelated to the formation of chromatid breaks. The cytogenetic approach used, combining CBMN with iFISH, is proposed as a valuable tool to test chemically induced asymmetric cell divisions.

1. Introduction

Caffeine is probably one of the most extensively studied naturally occurring dietary chemicals that have been linked both to beneficial and adverse health effects [1–11]. It occurs naturally in more than 60 plant species throughout the world and is one of the most frequently ingested neuroactive drugs to which people are exposed through food, beverages (e.g. coffee, tea, cola, energy drinks, chocolate) and medicines. The pharmacological effects of caffeine are known to be involved in cell cycle perturbation, programmed cell death or apoptosis, drugs toxicity enhancement and clastogenicity [2,9–23]. At cellular level, caffeine has a dual role: it affects the DNA structure and DNA synthesis (S-phase) through the intercalation of DNA [24], possibly via a local unwinding and, in parallel, at high concentrations, it abrogates G2/M cell-cycle checkpoint [25], allowing thus the damaged cells to proceed to the next phases of the cell cycle. Even though these effects could cause adverse cellular cancer related effects, the majority of references in the literature support that high concentrations of caffeine (>1 mM) are needed to reveal these effects. In addition, there is limited evidence for the carcinogenicity of caffeine (IARC, Group 3) in experimental animals and humans [24]. Particularly, there is lack of information concerning the potentiating mechanism of caffeine in cancer or cancer related cytogenetic effects [26,27].

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In contrast with the studies on the absence or even beneficial effects of caffeine [28] at low concentrations, numerous reports support that caffeine acts synergistically and modifies the mutagenic and carcinogenic potential of UV, ionizing radiation and chemical mutagens in various human and experimental models, increasing chemical and radiation risk [16,20–22,29–32]. Specifically, it has been shown that low concentrations of caffeine (0.1–10 μM) act synergistically when combined with ionizing radiation and increase in a dose dependent manner the radiation induced chromosomal damage, revealing the potential mutagenic profile of caffeine [33,34]. Interestingly, a very recent systemic review links caffeine intake during pregnancy with adverse birth outcomes [1]. Greenwood's et al. meta-analysis supports that consumption of caffeine is associated with increase in risk of spontaneous abortion (14% increase in risk), stillbirth (19% increase in risk), preterm delivery (2% increase in risk), low birth weight (increase in risk 7%) and small for gestational age (SGA) (increase in risk 10%) [1]. Strikingly, it is concluded that there is no identifiable threshold below which the associations are not apparent [1]. Given that chromosomal abnormalities and autosomal aneuploidies [35–37] are the major genetic etiologies of spontaneous abortions and adverse birth outcomes and also that caffeine is one of the most frequently ingested chemicals, the clastogenic and aneugenic effects of caffeine, particularly at low concentrations, need to be re-evaluated.

In the present study, we focus on the investigation of the cytogenetic effects of low concentrations of caffeine in vitro (i.e. <1 mM) in human peripheral blood lymphocytes (PBLs). For this purpose, we hypothesize that caffeine at low concentrations can also affect cellular DNA replication and cause chromosomal aberrations and asymmetric cell division but this effect cannot be easily detected at mitosis, as low caffeine concentration cannot abrogate the G2-checkpoint and the damaged cells are delayed during their G2/M transition. If this hypothesis is correct, the cytogenetic effects of caffeine at low exposure concentrations could be revealed when the damaged cells are forced to proceed to mitosis through the abrogation of their G2-checkpoint. Specifically, we used a 1 hour and 20 min 4 mM caffeine treatment for G2-checkpoint abrogation. Moreover, we tested the potential of low concentrations of caffeine to induce asymmetric cell divisions. To investigate whether the asymmetric cell divisions arise from misalignment of chromosomes of a normal parental cell, we evaluated the yield of caffeine-induced asymmetric cell divisions through the combination of the CBMN assay (cytokinesis-block micronucleus assay) with iFISH (interphase FISH) using the centromeric probes for chromosomes 11 and 17 (CEP11 and CEP17). The exposure of cells to cytochalasin-B prior to iFISH analysis ensures that the abnormal cells arise from the same parental cell and enables as well the visualization of the centromeric regions in micronuclei.

2. Materials and methods

2.1. Cultures of blood lymphocytes and caffeine exposures

Peripheral blood samples were taken by venipuncture from three healthy individuals and collected in heparinized tubes. Blood cultures were initiated by adding 0.5 ml of whole blood to each culture tube that contained 5 ml of RPMI medium, supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% antibiotics [penicillin (100 U/ml)- streptomycin (100 μg/ml)] and 2% phytohemagglutinin. All incubations were at 37 °C in a humidified incubator (5% CO_2, 95% air). All culture media were provided from Biochrom-AG, unless stated otherwise. Anhydrous caffeine (purity ≥99%, MW: 194.2) was purchased from Fluka. Caffeine was diluted in PBS and added, in blood culture, to reach a final concentration ranging from 0.01 μM to 10 mM. To test the effect of caffeine at lower concentrations, the following experimental procedure was applied: 48 h after whole blood culture initiation, the cells were exposed to a range of caffeine concentrations (0.01 μM-10 mM) for 24 h. Four hours after the addition of caffeine, a lower colcemid concentration (final concentration 0.05 μg/ml instead of 0.1 μg/ml), was added for 20 h to collect all the potentially damaged cells that will reach M-phase during this time period at mitosis. For G2/M checkpoint abrogation, caffeine treated cycling PBLs were exposed to 4 mM of caffeine and incubated for 1 hour and 20 min at 37 °C before fixation, as described previously by Terzoudi et al. [38,39]. The cultured cells were harvested by centrifugation (1300 rpm), treated with hypotonic KCl (75 mM) (Sigma–Aldrich) and fixed with freshly prepared 3:1 methanol-acetic acid (v/v) (purchased from Fluka and Baker, respectively).

2.2. Chromosomal aberration analysis

Cell suspension (20 μl) was dropped on wet slides. The slides were air dried and stained in a 3% solution of Giemsa dye (Merck) for 10 min and rinsed with water. Air-dried slides were embedded with cover slips and coded for analysis. For each experimental point, 300 well spread metaphases were analyzed for chromatid type aberrations and the total number of chromosomes by microscopic examination. To study the effect of 0.01 μM–4 mM caffeine on chromosome number of PBLs at the subsequent metaphase, we counted the chromosome number in 300 well spread metaphases and the percentage of cells with chromosome number 46, >46 and <46 was calculated. 2000 nuclei were also examined to determine the mitotic index (MI, i.e., percentage of cells undergoing mitosis). For each experimental point, mean values and standard deviations were obtained from three independent experiments. Data were evaluated statistically by Student's *t*-test. All *P* values were considered statistically significant at $P < 0.05$.

2.3. Cytokinesis-block micronucleus (CBMN) assay

The cytokinesis-block micronucleus assay (CBMN test) was performed using the cytochalasin-B technique described by Fenech and Morley [40]. At 44 h after peripheral blood culture initiation, cells were exposed to selected doses of caffeine (0.01 μM–10 mM) and were blocked in cytokinesis with the addition of cytochalasin-B (Sigma, St. Louis, MO; final concentration, 5.56 μg/mL). The total incubation time for all cultures was 72 h. The cells were harvested, fixed according to the protocol of Fenech [41] and stained for 10 min with 5% Giemsa (Merck). For each experimental point, about 2000 binucleated cells (BNC) were scored blindly, following the scoring criteria outlined by the HUMN Project [42], and the number of micronuclei per 2000 binucleated cells was recorded for each experimental point. Data were evaluated statistically by Chi-square test. All *p* values were considered statistically significant at $P < 0.05$. The nuclear division index (NDI) was calculated using the formula NDI = (M1 + 2M2 + 3M3 + 4M4)/n, where M1 to M4 indicates the number of cells with one to four nuclei, respectively, and n is the number of cells scored. To calculate the NDI, a minimum of 500 cells was also scored to determine the percentage of cells with 1, 2, 3 and ≥4 nuclei.

2.4Interphase fluorescence in situ hybridization (iFISH)

To evaluate the yield of caffeine-induced asymmetric cell divisions, we combined the CBMN assay with iFISH using the commercial probes CEP11 (D11Z1) SpectrumGreen Probe and CEP17 (D17Z1) SpectrumOrange Probe. DNA probes were applied following the standard procedures outlined by the manufacturer (Vysis Inc., Downers Grove, IL). A normal hybridization pattern for each cell consisted of two green (CEP11) and two red signals (CEP17), otherwise the pattern was considered abnormal. At least a total

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