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·*Original Article*·

Evidence of increased chromosomal instability in infertile males after exposure to mitomycin C and caffeine

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Abstract

Aim: To evaluate the genetic instability of 11 fertile and 25 infertile men. **Methods:** The methodology of sister chromatid exchanges (SCEs) was applied to cultures of peripheral blood lymphocytes, and the levels of SCEss were analyzed as a quantitative index of genotoxicity, along with the values of the mitotic index (MI) and the proliferation rate index (PRI) as qualitative indices of cytotoxicity and cytostaticity, respectively. The genotoxic and antineoplastic agent, mitomycin C (MMC), and caffeine (CAF) – both well-known inhibitors of DNA repair mechanism – were used in an attempt to induce chromosomal instability in infertile men, so as to more easily detect the probable underlying damage on DNA. **Results:** Our experiments illustrated that infertile men, compared with fertile ones, demonstrated a statistically significant DNA instability in peripheral blood lymphocytes after being exposed simultaneously to MMC and CAF. **Conclusion:** The current study showed vividly that there was genetic instability in infertile men which probably contributes to the development of an impaired reproductive capacity. *(Asian J Androl 2006 Mar; 8: 199–204)*

Keywords: male infertility; sister chromatid exchanges; mitomycin C; caffeine; chromosomal instability

1 Introduction

Infertility is both a private and a social problem. It is the inability of a couple to conceive, which is normally achieved by 80% of couples within a period of 12 months in which frequent, unprotected intercourse takes place [1]. Nowadays, about 10%–15% of couples face reproductive problems [1]. Studies have shown that infertility results from female disorders in 30%, male disorders in 30% and disorders in both partners in 30% [2]. There are also a number of cases (10%) where the etiology of infertility is unknown.

There is strong evidence that chromosomal and genetic abnormalities play a role in the development of male as well as female infertility [3]. Various syndromes, such as Klinefelter's (47,XXY), Noonan's (male Turner syndrome, 45,XO) and 47,XYY syndrome, manifest as numerical abnormalities in sex chromosomes and lead to infertility. In addition, a number of autosomal abnormalities, besides any other alterations in the phenotype, are also capable of leading to various forms of infertility. Such

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genetic disorders are cystic fibrosis, myotonic dystrophy and polycystic kidney disease [4, 5]. XX males, men with Kallman's syndrome (46,XY,delXp22.3), represent additional subpopulations of the infertile men. Since 1992, numerous studies have reported that microdeletions in one or more region (mainly in the long arm) of the Y chromosome are present in a frequency of about 8% of infertile men [6]. The phenotype of such men depends on the location and the extent of the deletion.

Previous studies on genome integrity and infertility indicated that an elevated rate of DNA nicks and double-strand breaks (DSBs) in human sperm could lead to infertility and 50% of miscarriages, which were unexplained, could be attributed to genetic abnormalities in the paternal genome [7]. Many mutated proteins, which possess a helicase activity or are otherwise involved in cell-cycle regulation and DNA-repair function, result in genetic disorders that present as genome instability [8, 9]. Such a protein encoded by the *BLM* gene is responsible for Bloom's syndrome [10]. The BLM protein is proposed to be associated with topoisomerases, while there is evidence for its involvement at late meiotic stages in resolving recombination events as a crossover control [10]. The correlation between the incidence of chromosome aneuploidies in sperm and somatic cells has also been demonstrated along with the possible role of mitotic instability in unexplained infertility [11].

The present study investigated the chromosomal instability of infertile men in comparison with fertile men. This was achieved by measuring a genotoxic index such as the sister chromatid exchanges (SCEs). SCEs are a natural phenomenon that are directly associated with DNA synthesis during the S phase of the cell cycle. SCE is defined as the exchange of genetic material between two sister chromatids before they separate during the metaphase. This exchange takes place in an unconditionally homologous locus, while its generation requires the breakage and the reunion of DNA chains of each sister chromatid**.** Studies have indicated that SCEs can be formed by the homologous recombination repair pathway, which is recruited to repair DSBs induced by certain DNA damaging agents such as ionizing radiation [9].

In a previous study of infertile individuals with idiopathic sterility, oligospermia and oligo-astheno-teratozoospermia, a significant increase in SCE frequencies was not evident [12]. Therefore, in order to expose the presumably increased DNA instability in infertile patients, we decided to use mitomycin C (MMC) and caffeine (CAF). Antineoplastic drugs generally enhance the frequency of SCE, and MMC is such a drug [13]. CAF elicits an increase in SCEs, which can be attributed to the fact that on one hand it induces the antineoplastic activity of alkyliotic factors and X-ray radiation because it suppresses the action of certain DNA repair mechanisms, on the other hand it enhances the genotoxic action of various agents *in vitro*. Studies have suggested that the frequency of SCE increases significantly along with chromosomal scissions in cultured cells when treated with CAF and MMC simultaneously [14]. The combined administration of these two substances made it easier to uncover the possible instability of the genetic material of the evaluated population through the induction of their SCE frequencies.

2 Materials and methods

Heparinized blood samples were obtained from 11 fertile men and 25 infertile men aged 28–50 years old, most of whom were volunteers from the Department of Urology at the Alexandroupolis University Hospital (Alexandroupolis, Greece). None of them was undertaking any drug treatment. The fertile group consisted of men who had one or more children. The infertile group consisted of men who faced reproductive problems. The latter group included men with an abnormal sperm diagram illustrating various forms of infertility, such as azoo-spermia, aspermia, necrospermia, oligospermia, hyper-spermia, asthenospermia, oligo-astheno-teratozoospermia (56%) and mainly oligo-asthenozoospermia. Many of these men were also found to present with varicocele (43%).

Cultures of peripheral blood lymphocytes were prepared in universal containers by adding 11 drops of whole blood to 5 mL of Chromosome Medium B (Biochrom KG, Berlin, Germany). These were incubated at 37ºC for 72 h. Cultures were treated with MMC (12 ng/mL culture medium) and CAF (120 μg/mL culture medium) and cells were allowed to proliferate for 2 mitotic cycles in the presence of BrdU at a final concentration of 6 μg/mL. After 70 h, colcemide was added for 2 h and at the end of the incubation period cultures were harvested. Cultures were maintained in the dark to prevent or minimize the photolysis of BrdU. Chromosome preparations were stained using the fluorescence plus Giemsa technique [15].

Scoring was performed in a blind fashion. Cells on the first, second, third and subsequent mitotic divisions were counted. Three indices were evaluated: 1) the mi-

totic index (MI), which is a qualitative index of cytotoxicity; 2) the proliferation rate index (PRI), which is a qualitative index of cytostaticity; and 3)SCEs, which is a qualitative and quantitative index of genotoxicity. All indices were evaluated in each subject for either treatment. Mean SCE values were evaluated only in the suitable second division metaphases, because only in these metaphases we were able to observe and count SCEs. In order to establish the PRI, 200 cells were counted and the following formula was used: PRI = $(M_1 + 2M_2 + 3M_{3+})/N$, where M_1 is the percentage of cells in the first division, M_2 in the second and M_{3+} in the third and subsequent divisions, while N is the total number of cells counted (i.e. $(M_1 + M_2 + M_{3+})$). The MI for 5 000 activated lymphocytes was determined for all cultures as mentioned before.

To compare various treatments, a logarithmic transformation of the SCE values was performed using one-way analysis of variance (ANOVA) and the Duncan test for pair-wise comparisons. The evaluation of MI and PRI was based on the χ^2 test [15, 16]. A probability of $P < 0.05$ was considered statistically significant.

3 Results

3.1 The effects of MMC and CAF treatment on fertile men

CAF and MMC elicited 56.41% and 58.02% increase in SCE frequency in fertile individuals, respectively. The simultaneous administration of these substances gave rise to a statistically significant rise in the SCE frequency: there was a 2-fold increase compared to that in controls (Table 1).

With regard to the MI, MMC alone had a smaller cytotoxic effect ($P<0.05$) than CAF, which reduced the number of mitotic divisions at a percentage of 51.47% ($P<0.01$) compared to the control. The acute cytotoxic activity of CAF was exhibited in cultures that were incubated with both agents simultaneously ($P< 0.01$) (Table 1).

The PRI illustrated statistically significant changes when the cells were treated with MMC and/or CAF ($P < 0.01$) compared to the control. It seems that CAF enhanced the cytostatic activity of MMC as was illustrated in the combined treatment ($P < 0.05$ compared to MMC alone) (Table 1).

3.2 The effects of MMC and CAF treatment on infertile men

CAF treatment elicited a 46.42% increase in the SCE frequency in infertile individuals. MMC on the other hand led to a 79.57% increase in the SCE levels of the same group compared to the control. Simultaneous treatment with MMC and CAF contributed to a spectacular, statistically significant ($P < 0.01$) result, increasing SCE frequency by 2.85 times compared to that in the control (Table 2).

MMC alone caused a negligible, statistically insignificant increase in MI (1.38%). In contrast, CAF gave rise to a statistically significant reduction ($P < 0.01$) in the number of cell divisions (a 38.97% decrease) compared to the controls. The acute cytotoxic activity of CAF was exhibited in cultures treated with the combination of these two agents ($P < 0.01$, compared to all other

Table 1. Details of the cytogenetic damage done by mitomycin C (MMC) to the caffeine-exposed cultured human lymphocytes of fertile men. The sister chromatid exchanges (SCEs) frequency was based on 20–25 second generation metaphases for each donor and either treatment; for proliferation rate index (PRI), 200 cells were counted and for mitotic index (MI), 5 000 activated lymphocytes, for each donor and either treatment for both indices. The results were based on 11 experiments from 11 donors. PRI and MI comparisons were made using the χ^2 test. For SCE comparisons, logarithmic transformation of the data was performed using one-way ANOVA and the Duncan test. *If EV = expected value and OV = observed value, then EV = 14.71 where, $EV_{(MMC + CAF)} = OV_{(CAF)} + OV_{(MMC)} - OV_{(control)}$ or better, $EV_{(MMC + CAF)} = OV_{(CAF)} + OV_{(MMC)} - OV_{(control)}$, [a]$P < 0.01$ *vs.* line 1; [b]$P < 0.01$ *vs.* lines 1, 2 and 3; [c]$P < 0.05$ *vs.* line 1; [d]$P < 0.01$ *vs.* lines 1 and 2, [e]$P < 0.05$ *vs.* line 2; CAF, caffeine.

Agent (concentration)	Mean SCEs ± SEM (range of values)	MIP(‰)	Percentage of cells in first, second, or subsequent (third +) divisions			
			1st	2nd	3rd+	PRI
Control	16.86 ± 0.29 (1.0-22.3)	28.89	5.5	17.0	77.5	2.72
MMC (12 ng/mL)	10.84 ± 0.42[a] (1.0-32.4)	27.75 [c]	5.5	21.5	73.0	2.67[a]
CAF (120 μg/mL)	10.73 ± 0.41[a] (2.0-25.7)	14.02 [d]	7.5	24.5	68.0	2.60[d]
MMC+ CAF	14.24 ± 0.52[b] (3.0-48.2) *(EV=14.71)	14.25 [d]	7.0	24.0	69.0	2.62[a, e]

cultures) (Table 2).

The cytostatic effects of MMC ($P < 0.05$ compared to the control) and CAF ($P < 0.01$ compared to the control) were demonstrated by the reduction of the PRI, while the combined treatment demonstrated a statistically significant decrease as well ($P < 0.01$ compared to all other treatments) (Table 2).

4 Discussion

The value and the significance of SCEs rest in the fact that their evaluation could solve problems that concern chromosome structure and function, as well as DNA transcription. They are mainly used to study the harmful action of several physical or chemical agents on DNA. Furthermore, it has been proved that there is a linear relationship between SCEs and mutations [16]. This particular assay can be used as a means to quantitate genetic instability. In order for eukaryotic cells to overcome the potentially mutagenic and cytotoxic properties of DSBs caused by DNA-damaging agents but also spontaneously arising DNA damage, various repair pathways are employed along with homologous recombination. In this particular pathway, sister chromatids play a crucial role [17]. This is because cohesion proteins hold together sister chromatids during the G_2 phase, and something that enables recombination events to take place. On the other hand, the fact that another DNA repair pathway, or mismatch repair pathway, prevents recombination between homologous sequences might actually be the reason why recombination between homologous chromosomes is prevented [9].

The genetic background that could lead to infertility varies, including structural and numerical chromosomal abnormalities. Since such major abnormalities are present in cells, it might be probable that other "minor" genetic abnormalities are present, too. In addition, it has been reported that such "minor" damage is present in sperm and since there is a correlation between sperm and somatic cells (as far as the incidence of chromosomal aneuploidies is concerned), this particular study attempted to determine through the SCE assay, the stability of the DNA of infertile men and how susceptible it is to exogenous DNA-damaging agents [7, 11].

As far as our results are concerned, the infertile group illustrated increased instability – infertile men were more inclined to have induced DNA damage because their SCE levels exhibited an almost 3-fold increase when cells were treated with CAF and MMC simultaneously, which was higher than the expected value (see EV, Table 2). The SCE frequency in fertile men also, as expected, increased, but this increase was within the range of our expectations and was consistent with previous reports [14]. Feedback control operates at the mitotic entry checkpoint to prevent cells with damaged DNA from entering mitosis until the damage is repaired. MMC is a cross-linking clastogen. Induced by MMC, cross-links lead to SCE formation through a cross-link bypass model rather than homologous recombination, which has been proposed as one of the mechanisms of SCE formation [18]. On the other hand, CAF enables cells to undergo mitosis and shortens the G_2 phase, preventing DNA repair to be completed. It particularly affects DNA pre-transcriptional repair (excision repair) while it can also suppress the gap

Table 2. Details of the cytogenetic damage done by mitomycin C (MMC) to the caffeine (CAF)-exposed cultured human lymphocytes of infertile men. The sister chromatid exchanges (SCEs) frequency was based on 20–25 second generation metaphases for each patient and either treatment; for proliferation rate index (PRI), 200 cells were counted and for mitotic index (MI), 5 000 activated lymphocytes for each patient and either treatment for both indices. The results were based on 25 experiments from 25 patients. PRI and MI comparisons were made using the χ^2 test. For SCE comparisons logarithmic transformation of the data was performed using one-way ANOVA and the Duncan test. *If EV = expected value and OV = observed value, then EV = 14.71 where, $EV_{(MMC + CAF)} = OV_{(CAF)} + OV_{(MMC)} - OV_{(control)}$ or better, $EV_{(MMC + CAF)} = OV_{(CAF)} + OV_{(MMC)} - OV_{(control)}$, [a]$P < 0.01$ *vs*. line 1; [b]$P < 0.05$ *vs*. line 1; [c]$P < 0.01$ *vs*. lines 1 and 3; [d]$P < 0.05$ *vs*. line 2, [e]$P < 0.01$ *vs*. lines 1, 2; [f]$P < 0.01$ *vs*. lines 1, 2 and 3.

Agent (concentration)	Mean SCEs ± SEM (range of values)	MIP(‰)	Percentage of cells in first, second, or subsequent (third +) divisions			
			1st	2nd	3rd+	PRI
Control	5.58 ± 0.19(1.0-20.0)	22.53	6.0	15.5	78.5	2.73
MMC (12 ng/mL)	10.02 ± 0.33[a] (2.0-39.0)	22.84	5.0	17.0	78.0	2.73[b]
CAF (120 µg/mL)	8.17 ± 0.24[b] (1.0-37.5)	13.76[e]	5.5	19.0	75.5	2.70[a,d]
MMC+ CAF	15.91 ± 0.51[c,d] (2.0-43.6) *(EV=12.61)	12.45[f]	7.5	25.5	67.0	2.60[f]

filling process [19]. Thus, it would not be too arbitrary to point out that the reason of this synergistic action is due to the fact that MMC-induced SCEs and CAF-induced SCEs are attributed to different mechanisms. If this were not the case, we would expect the levels of SCEs in the combined treatment to be less than or at least similar to those induced by the MMC treatment alone.

The fact that CAF treatment elicited a higher increase in SCE frequency in fertile men than that in infertile ones could be because the types of spontaneously arising damages present in fertile and infertile individuals are different.

CAF enables spontaneous damage to bypass checkpoint controls, thus justifying its high cytotoxic action. The intense cytotoxic properties of CAF were obvious by the reduction of the MI in both groups, while MMC treatment proved to be cytotoxic only in fertile subjects. This can be attributed to the fact that normal, healthy individuals whose genetic material has not undergone any crucial alterations are more responsive to cytotoxic agents. Finally, it seems that the cytotoxic activity of CAF enhanced the activity of MMC, when these were introduced concomitantly to cells. The combined treatment gave rise to increased levels of cytotoxicity. Since MMC induces DNA damage and CAF inhibits some DNA repair pathways, the impairments caused by MMC can lead to cell death [16]. CAF and MMC also influenced the PRI significantly in both examined groups.

This study describes the especially fragile nature of the genetic material of infertile men in comparison with fertile men. The DNA of infertile individuals demonstrated a particularly increased instability, which could be connected with structural and functional rearrangements. As in the case of Bloom's syndrome, where the mutated protein BLM plays a role in meiotic recombination (whereas it normally prevents SCEs in somatic cells and stabilizes chromosome structure), we could hypothesize that something similar takes place generally in infertile subjects [10]. Infertility could be associated with BLM protein or another protein with similar properties or even other proteins that participate in cell-cycle regulation and repair pathways. These are only speculations but it would be interesting to further investigate this particular area further. In a different study, Wenger [20] noticed that MMC has the ability to induce SCEs at fragile sites that are more prone to single-strand nicks, which could be related to our findings concerning the type of spontaneous DNA lesions present in infertile individuals. Thus, genetic instability could be attributed to an increased tendency of fragile sites to break something that might also lead to chromosomal aberrations.

The results of the present study do not fully explain the etiology of the impaired reproductive potential of infertile men, but they contribute to the completion of the "puzzle". However, we can verify the possible correlation of reproductive problems with DNA cell cycle regulation defects. This fact might be of great clinical and therapeutic value in the future, even if more in-depth studies are desirable.

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