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Chromosome analysis of mouse zygotes after injecting oocytes with spermatozoa treated in vitro with green tea catechin, (-)-epigallocatechin gallate (EGCG)

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Title: Chromosome analysis of mouse zygotes after injecting oocytes with spermatozoa treated *in vitro* with green tea catechin, epigallocatechin gallate (EGCG)

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Abstract

The cytogenetic effects of (-)-epigallocatechin gallate (EGCG) on mouse spermatozoa were studied *in vitro* using an intra-cytoplasmic sperm injection (ICSI) technique. Spermatozoa were collected by the swim-up method and treated with EGCG at 1 μM and 10 μM. When motile, EGCG-treated spermatozoa were injected into oocytes, structural chromosome aberrations at the first cleavage metaphase did not increase significantly. However, a majority of immotile spermatozoa treated with 10 μM EGCG had the following abnormalities: pronuclear arrest (11% of activated oocytes), degenerated sperm chromatin (chromosome) mass (30% of activated oocytes) and occurrence of structural chromosome aberrations (57% of analyzed metaphases). The incidence of these abnormalities suggests that immotile spermatozoa were susceptible to EGCG, and that the damage of sperm chromatin was accelerated in immotile spermatozoa by 10 μM EGCG treatment.

1. Introduction

Green tea catechin, (-)-epigallocatechin gallate (EGCG), has potentially positive effects on human health as suggested by *in vitro* studies. These reports concluded that EGCG prevents spontaneous mutations [1], chromosomal damage induced by reactive oxygen species (ROS) in somatic cells [2,3] and growth of cancer cells [3-6]. However, EGCG also induces various kinds of chromosomal damage in cultured cells treated at levels above 10 μM [2,7]. Those levels are non-physiologically high compared with EGCG in human blood and urine where concentrations are approximately 1 μM [8]. EGCG treatment can also damage both isolated and cellular DNA [9,10].

In this study, we evaluated whether 1 μM and 10 μM EGCG induced chromosomal damage in mouse spermatozoa. In addition, we examined whether there were differences in the effects of EGCG on motile and immotile spermatozoa. We found several deleterious changes which suggest that 10 μM selectively promotes deterioration of immotile sperm.

2. Materials and methods

2.1. Animals

B6D2F_1 hybrid male and female mice, 7-12 weeks of age, were purchased from

Sankyo Labo Service, Co. (Sapporo, Japan). They were maintained under a 14 h light/10 h dark photoperiod at a room temperature of 22-24^{o}C. Food and water were provided *ad libitum*.

2.2. Chemicals and injection medium

All chemicals were obtained from Nakalai Tesque (Kyoto, Japan) unless otherwise stated. (-)-Epigallocatechin gallate (EGCG, CAS No.: 989-51-5) was purchased from Sigma (St. Louis, MO, USA). The medium for preparation of oocytes and sperm injection was HEPES-CZB medium [11], modified with 20 mM HEPES, 5 mM $NaHCO_3$, and 0.1 mg/ml polyvinyl alcohol (PVA; cold water soluble; molecular weight: 30,000-50,000; Sigma, St. Louis, MO, USA) instead of BSA [12].

2.3. Sperm collection and treatment with EGCG

Both cauda epididymes were removed from males and punctured with sharp forceps. A dense mass of spermatozoa was squeezed out of the cauda region and placed in a 1.5 ml polypropylene centrifugation tube containing 0.5-1.0 ml of HEPES-CZB medium. The tube was left standing for 10 min at 37^{o}C to allow sperm to swim up. After 0.2-0.5 ml of the sperm suspension was collected, it was placed into another 1.5 ml

polypropylene tube.

EGCG was dissolved in distilled water at 200 and 2000 μM. Distilled water (for solvent control) or one of the EGCG solutions was added to the sperm suspension at 0.5% of the total volume. This yielded final concentrations of 1.0 μM and 10.0 μM EGCG in which the sperm were incubated for 30 min at 37^{o}C.

2.4. Intracytoplasmic sperm injection (ICSI)

ICSI was carried out as previously described and modified [11, 13]. All operations were performed at room temperature (18-25^{o}C). To collect oocytes, female mice were superovulated with an i.p. injection of 10 units of pregnant mare's serum gonadotrophin (Teikokuzouki, Tokyo, Japan) and 10 units of human chorionic gonadotrophin (hCG, Mochida, Tokyo, Japan) given 48 h apart. Oocytes were collected from oviducts between 13 h and 16 h after hCG injection. The cumulus-intact oocyte mass was treated with 0.1% hyaluronidase dissolved in HEPES-CZB medium to remove the cumulus cells. Cumulus-free oocytes were placed in droplets of HEPES-CZB medium.

The sperm suspension (1-5 μl) was thoroughly mixed with one drop (5-10 μl) of HEPES-CZB medium containing 10% polyvinylpyrrolidone K-90 (HEPES-CZB-PVP). Motile and immotile spermatozoa were differentiated under an inverted microscope

(IX70, Olympus, Tokyo, Japan) at a magnification of 200. The spermatozoa that were not moving were defined as immotile. Motile and immotile spermatozoa were selected for ICSI concurrently for each treatment group. The sperm heads were separated from the midpiece and tail by pipetting with an injection pipet and applying one or more piezoelectric pulses [11]. The sperm heads were injected into oocytes within 1 h after suspension in HEPES-CZB-PVP. ICSI was repeated 4 to 7 times in each treatment group.

2.5. Culture of oocytes injected with spermatozoa

Sperm-injected oocytes were transferred into droplets (50-100 μl) of the modified CZB medium [12] supplemented with 5.56 mM glucose and 5 mg/ml BSA (fraction V, Sigma, St. Louis, MO, USA). Then the oocytes were cultured under a paraffin oil (Merck, Dermstadt, Germany) overlay in a humidified atmosphere of 5% CO_2 in air.

2.6. Chromosome analysis

Chromosome specimens of ICSI-treated oocytes were prepared at the first cleavage metaphase. At 5-6 h after ICSI, oocytes were placed into a modified CZB medium containing 0.006 μg/ml vinblastine to arrest the metaphases of the first cleavage. After

treatment with vinblastine for 16-18 h, the zona pellucida was removed with 0.5% protease (1000 units/mg, Kaken Pharmaceuticals, Tokyo) before placing the oocytes in a hypotonic citrate solution (1:1 mixture of 30% FBS and 1% sodium citrate) [14,15]. Fixation of oocytes and spreading of chromosomes onto glass slides were performed according to the methods described previously [16].

Structural chromosome aberrations (SCAs), i.e., chromatid and chromosome breaks and exchanges, were scored as outlined previously [17]. Uncountable numbers of structural aberrations such as chromosome fragmentation and multiple exchanges were arbitrarily assigned 10 SCAs per zygote. The number of SCAs per metaphase plate was recorded without discriminating between paternal (sperm) and maternal (oocyte) pronuclear chromosome sets.

2.7. Analysis of data

Statistical comparisons of SCAs between control and experimental groups were made with the chi-square test using Yates' correction for continuity.

3. Results

The effect of EGCG on sperm motility was examined three times in preliminary

experiments. Frequencies of motile spermatozoa were 58.6±9.1%, 61.6±6.8%, and 56.7±14.1% in 0, 1 and 10 μM EGCG, respectively. Thus, the adverse effect of EGCG on the sperm motility was not significant at concentrations up to 10 μM. The ability of motile and immotile spermatozoa to activate oocytes was maintained at greater than 90% after treatment with EGCG (Table 1).

In solvent controls, the frequency of zygotes with SCAs (Table 1) was higher when immotile spermatozoa (26%) were compared to motile spermatozoa (5.5%). These frequencies were similar to values previously reported [13]. When oocytes were injected with motile spermatozoa treated with EGCG, the frequency of zygotes with SCAs (Table 1) and the number of SCAs per metaphase plate (Fig. 1) showed no increase up to 10 μM. However, when oocytes were injected with immotile spermatozoa, the frequency of zygotes with SCAs increased significantly ($P < 0.01$, Table 1, Fig. 2a) after treatment with EGCG at 10 μM (57%), but not at 1 μM (41%). Additionally, the number of SCAs per metaphase plate showed a dose-dependent increase (Fig. 1).

At 10 μM EGCG, 30% of oocytes injected with immotile spermatozoa contained degenerated sperm chromatin or chromosomes (Table 1, Fig. 2b-e). All of them were accompanied by one normal metaphase plate originating from the female pronucleus (Figs. 2b). At the same concentration, pronuclear arrest was also significant ($P<0.05$,

Table 1).

4. Discussion

EGCG did not affect the sperm motility at the concentration of 10 μM. Thus, spermatozoa that have already lost motility must be more sensitive to EGCG than the motile spermatozoa. Clastogens are known to induce chromosomal damage in motile spermatozoa used in *in vitro* fertilization [18]. In this study, since EGCG did not induce chromosomal damage in motile spermatozoa, we conclude that EGCG had no direct injurious effect on sperm DNA at up to 10 μM. However, the number of SCAs per metaphase plate increased in immotile spermatozoa after treatment with at 1 μM and 10 μM EGCG (Fig. 1). We have reported that immotile spermatozoa suspended in HEPES-CZB accumulated chromosomal damage after preservation for 1 day [13]. It seems that a part of the sperm population tends to accumulate chromosomal damage after the short-term (30 min) treatment with EGCG even at the physiological concentration of 1 μM,

Degenerated sperm chromatin mass would fail to form the male pronucleus or chromosomes (Fig. 2b-e). There is a possibility that degenerated sperm chromatin mass and pronuclear arrest are caused by the deterioration of sperm plasma membrane

or sperm chromatin after EGCG treatment. While EGCG can protect cell membranes from lipid peroxidation [19,20], it can also act as a pro-oxidant. At concentrations higher than physiological levels, it produces hydrogen peroxide [2,10,21] that damages DNA and sperm membrane via lipid peroxidation [22,23]. Also, it inhibits sperm chromatin decondensation induced by dithiothreitol in human sperm [24]. Accordingly, in our study, the degenerated sperm chromatin mass could be the result of sperm membrane damage induced by ROS produced from EGCG.

This study demonstrated that pronuclear arrest, degenerated sperm chromatin mass and SCAs were induced in immotile spermatozoa after treatment with EGCG at 10 μM. Our results suggest that EGCG tends to act selectively on immotile spermatozoa. Further studies are necessary to address the mechanism(s) of sperm deterioration enhanced by EGCG.

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Table 1

Chromosome analysis of oocytes injected with mouse spermatozoa treated with (-)-epigallocatechin gallate (EGCG) for 30 min in vitro

Sperm	EGCG (μM)	No. (%) oocytes activated [1]	No. zygotes for chromosome slide	No. of zygotes with		
				Pronucleus arrest (%)	Degenerated sperm chromatin mass [2]	SCAs [3] / total metaphase plates analyzed (%)
Motile	0	115 (98)	91	0(0.0)	0(0.0)	5/91(5.5)
	1	91 (97)	74	1(1.4)	1(1.4)	6/72(8.3)
	10	142 (97)	106	3(2.8)	1(0.94)	5/102(4.9)
Immotile	0	102(100)	79	1(1.3)	1(1.3)	20/77(26)
	1	105(98)	86	4(4.7)	1(1.2)	33/81(41)
	10	119 (94)	105	12(11)*	32(30)***	35/61 (57)**

Significantly different from solvent control (*P<0.05, **P<10^{-3}, ***P<10^{-6}).

[1] Activation of oocytes was judged by the presence of a second polar body and female pronucleus. [2] Includes non-decondensed sperm head. [3] Structural chromosome aberrations.

Legends of Figs.

Fig. 1. Increase in SCAs in zygotes produced by motile and immotile spermatozoa treated with (-)-epigallocatechin gallate (EGCG). EGCC increased the number of SCAs per metaphase plate in immotile spermatozoa but not in motile ones.
*****The metaphase plate consists of paternal and maternal chromosome sets that have been analyzed for the chromosomal aberration assay.

Fig. 2. Structural chromosome aberrations and degenerated sperm chromatin mass observed at the first cleavage metaphase of mouse oocytes after injection with immotile spermatozoa treated with 10 μM EGCG. (a) A chromosome fragment (left arrow) and a chromosome exchange (right arrow) (b) A normal metaphase plate of maternal origin (right) and a degenerated sperm chromatin mass (left); (c) Un-decondensed sperm head, representing a sperm head with a shape similar to an intact sperm head; (d) A sperm head with a rough outline; (e) A pulverized sperm head. Sperm heads seen in (b-e) were recorded as the "degenerated sperm chromatin (chromosome) mass". The scale bar represents10 μm.

Fig. 1

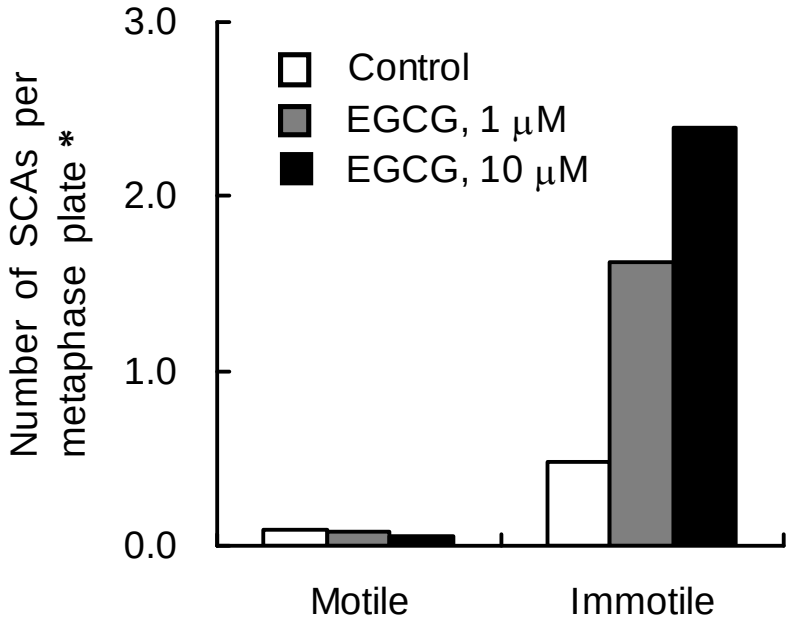
3.0
2.0
1.0
0.0
Number of SCAs per metaphase plate *
Control
EGCG, 1 μM
EGCG, 10 μM
Motile
Immotile

Fig. 2