e-ISSN: 2177-5133
www.ufpi.br/comunicata

Comunicata Scientiae 5(3): 213-221, 2014

Original Article

Modulatory Effect of Green Tea against Genotoxicity Induced by Hydrocortisonein Mice

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Abstract

Hydrocortisone (corticosteroid, steroid) drug used for treatment of a variety of inflammatory, asthma, allergic and immune diseases. The widespread use of this drug is of great concern to human health problems. Therefore, the genotoxic potential of hydrocortisone was evaluated using different mutagenic endpoints including sister chromatid exchanges (SCEs) in bone marrow cells and chromosomal aberrations (CAs) in mouse spermatocytes. The modulatory effect of the methanol extract of green tea (MEGT) was also studied. Hydrocortisone was given intraperitoneally (i.p) at three doses 26, 39 and 52 mg/kg b.w. MEGT was given orally at dose 390 mg/kg. Mice were simultaneously treated with hydrocortisone and MEGT in single treatment and repeated treatment. The results showed that all doses of hydrocortisone induced significant increase in the frequency of SCE's with a dose dependent manner. Both Single and repeated treatment of hydrocortisone induced a statistically significant in the incidence of chromosomal aberration in mouse spermatocytes excepting the dose 26 mg⁄ kg b.w in single treatment regimen. These findings suggested that hydrocortisone has genotoxic activity in bone marrow and spermatocytes. On the other hand, MEGT suppressed the frequency of SCEs andCAs induced by hydrocortisone however such suppressionwasnot reached to the normal control values.

Key words: Chromosomal aberration, Hydrocortisone, Green tea, Mice, Sisterchromatid exchange

Efeito modulador do chá verde contra genotoxicidade induzida por Hydrocortisonein Ratos

Resumo

A hidrocortisona (corticosteróides, esteróides) é uma droga usada para o tratamento de uma variedade de doenças inflamatórias, asma, alergia e doenças imunológicas. O uso generalizado desta droga é de grande preocupação, para os problemas de saúde. Portanto, o potencial genotóxico da hidrocortisona foi avaliada utilizando diferentes "end points" mutagénicos incluindo trocas de cromátides irmãs (SCE) em células da medula óssea e aberrações cromossômicas (AC) em espermatócitos de mouse. O efeito modulador do extrato metanólico de chá verde (MEGT) também foi estudada. A hidrocortisona foi administrado por via intraperitoneal (ip) em três doses 26, 39 e 52 mg / kg pc MEGT foi dado oralmente na dose de 390 mg / kg. Os ratos foram tratados simultaneamente com hidrocortisona e MEGT em um tratamento único e tratamento repetido. Os resultados mostraram que todas as doses de hidrocortisona induziram aumento significativo na freqüência de SCE com uma forma dependente da dose. Tanto o tratamento única e repetida de hidrocortisona induziu um aumento estatisticamente significativo na incidência de aberrações cromossómicas em espermatócitos rato com exceção da dose de 26 mg / kg em regime único. Estes achados sugerem que a hidrocortisona tem atividade genotóxica na medula óssea e de espermatócitos. Por outro lado, a frequência suprimida MEGT da SCE e CAs induzidas pelo uso de hidrocortisona no entanto, a sua supressão não foi alcançado com os valores de controlo normais.

Palavras-chave: Aberração cromossômica, Hidrocortisona, Chá verde, Camundongo, Troca de cromátides Irmãs

Received: 29 January 2013
Accepted: 26 Maio 2013

Introduction

In recent years, there has been an increasing awareness of the genotoxic potential of a wide variety of chemicals to which the human population is exposed whether environmentally or occupationally. As there is a need to combat human diseases, many drugs are being used in the medical field. Such drugs when used in high doses they may be toxic to the genetic system of human beings. The mutagenicity studies of drugs employing short term tests in mammals, so they have received much attention as a protective prerequisite to human life (Custer &Sweder 2008).The screening of new drugs for potential genotoxicity is an important step during research and development. Steroid hormones are active biological agents that function as natural hormones**,** chemical ligands, and precursors for endogenous synthesis of other chemicals. They are secreted by the adrenal cortex, testis, ovary, and placenta and include the progestogens, glucocorticoids, mineralocorticoids, androgens, and estrogens (Rubin, 1982).Hydrocortisone, synthetic preparation of steroid hormone cortisol, was chosen for our experiments with mice for several reasons:1) it is widely used for treatment of a variety of inflammation, asthma, allergic, cancer and immune diseases (Sweetman, 2009); 2) very few data are available concerning the genotoxic hazard of hydrocortisone in mice.

Tea (*Camellia sinensis*), a tropical shrub originated in Southern China, is consumed by over two thirds of the world's population. It is the most widely used beverage in the world next to water(Thakur et al., 2012). Green tea constitutes 20 % of the tea manufactured worldwide and the rest 80% is black and oolong tea. The polyphenols or catechins are the most important chemicals present in green tea leaves with considerable pharmacological significance and account for 25-35% of their dry weight. Among the most extensively investigated and well-defined dietary chemopreventives, polyphenol (–) -epigallocatechin-3-gallate (EGCG) is a principal antioxidant contained in green tea and reported to have multiple health benefits (Thakur et al., 2012).Numerous EGCG studies demonstrated chemopreventive and chemotherapeutic actions in cellular and animal models of cancer (Yang et al., 2009). Tea polyphenol has selective proapoptotic activity in transformed cells (Borska et al., 2003) and affects all stages of the carcinogenicprocess i.e. inhibit enzyme activities and signal transduction pathways, resulting in the suppression of cell proliferation and enhancement of apoptosis, as well as the inhibition of cell invasion, angiogenesis and metastasis (Kuroda& Hara, 1999;Yang et al., 2009). Moreover, tea drinking has recently proven to be associated with cell-mediated immune function of the human body(Wu et al., 2012)and protects against hepatitis C virus entry(Calland et al., 2012).Earlier studies of the antimutagenic activity of green tea were to be effective in reduction chromosomal aberrations (CAs), micronucleated polychromatic erythrocytes, sister chromatid exchange (SCEs), gene forward mutation induced by various chemicals in both in vitro and in vivo system (Bhattacharya& Girl, 2012; Ito et al., 1989;Wang et al., 1989;Imanishi et al., 1991). A remarkable reduction in micronuclei and SCEs in blood peripheral lymphocytes was shown in cigarette smokers who consumed green tea (2-3 cups per day) for six months (Lee et al., 1997; Shim et al al., 1995).

Considering this, the present study was carried out to study the hypothesis that hydrocortisone induced genotoxic effect in mice using SCEs in mouse bone marrow cells and CAs in mouse spermatocytes. In addition, we have analyzed the modulatory effects exerted by methanol extract of green tea (MEGT) against the hydrocortisone induced genotoxicity in mice.

Material and Methods

Animals

Male white Swiss mice (*Mus musculus*), aged 9-12 weeks were used in all experiments. The animals were obtained from a closed random bred colony at the National Research Center, Dokki, Cairo;Egypt. The mice used for the experiments were selected from mice of similar age (± 1 week) and weight (± 2g). Animals were housed in polycarbonate boxes with steel-wire tops (not more than five animals/ cage) and bedded with wood shavings. Ambient temperature was controlled at 22± 3ºC with a relative humidity of 50 ± 15% and a 12- h light/dark

photoperiod. Food and water were provided ad *libitum*. Animals were sacrificed after treatment by cervical dislocation. The study protocol was approved by the Ethics Committee for Animal Care of National Research Center, Dokki, Cairo, Egypt.

Test substances

Solu-Cotef® (Hydrocortisone sodium succinate) manufactured by Egyptian Int. Pharmaceutical Industries Co. (EIPICO), under the license of UPJOHN,Puuurs- Belgium. Hydrocortisone was given intraperitoneally (i.p) to mice at three doses 26, 39 and 52 mg/kg b.w.According to the formula of Paget and Barnes (1964), these doses were equivalent to the recommended therapeutic doses of hydrocortisone for human which are 200,300,400 mg/adult (70kg) respectively.

Endoxan® (Cyclophosphamide monohydrate) manufactured by Baxter oncology GmbH, Frankfurt–Germany. Cyclophosphamide (CP) was used as positive controlat the dose of 20 mg/kg b.w.

Plant extraction and dosage

Green tea leaves (*Camellia sinensis*) obtained from the Department of Aromatic and Medicinal Plants, National Research Center, Cairo, Egypt. Dry green tea leaves (53.69g) were ground with a mill. Green tea covered with methanol and distilled water (1:1) in round bottom flask equipped with a condenser, and left for 3 days at room temperature. The process of extraction was repeated 3 times. Methanol evaporated under vacuum at 60◦C. Water evaporated at 100◦C. The extraction residue obtained (28.5g) was dissolved in distilled water and constantly stirred with magnetic stirrer (Perva-Uzunalic et al., 2006).According to the formula of Paget&Barnes (1964), MEGT was given orally to mice at the dose 390 mg/kg b.w which equivalent to 3g of green tea per adult human.

Treatment schedule:

Two kinds of treatment were given to mice 1) Single treatment, 2) repeated treatment were given for successive 7 days as shown below.

Treatment group	*Treatment schedule*
I- Negative control	No treatment (distilled water)
II- Positive control (CP)	Mice given single i.p injection of CP at 20mg/kg b.w
III -Single treatment	
1- Hydrocortisone alone	Three groups of mice were given i.p injection (0.1 ml) of hydrocortisone at three different doses 26, 39, 52 mg/kg b.w
2- Negative Extract (MEGT) alone	Mice given orally MEGT (0.1ml) at the dose 390 mg/kg
3- Hydrocortisone +MEGT	Three groups of mice were simultaneously treated with hydrocortisone (i.p.) at the doses 39 and 52 mg/kg b.w and MEGT at 390 mg/kg b.w
IV-Repeated treatment for 7 days	
1. Hydrocortisone alone	Three groups of mice were given i.p injection (0.1 ml) of hydrocortisone at three different doses 26, 39, 52 mg/kg b.w for successive 7 days
2- Negative Extract (MEGT) alone	Mice were orally administrated successive daily doses of MEGT at 390 mg/kg b.w for 7 days
3- Hydrocortisone +MEGT	Three groups of mice were simultaneously treated with hydrocortisone (i.p.) at the doses 26, 39 and 52 mg/kg b.w and MEGT at 390 mg/kg b.w (oral) for successive 7 days.

Sister chromatid exchange assay

SCE assay is a short-term test for the detection of reciprocal exchanges of DNA between two sister chromatids of a duplicating chromosome.The experimental procedure was conducted following the protocol of Allen (1982) using single exposure of test substance. Briefly, tablets of 5-Bromodeoxyuridine (5-BrdU) weighing approximately 55±2mg, were implanted subcutaneously in the lower lateral region of each mouse under mild anesthesia and 30 min test substance was injected to mice. Following 5-Brdu treatment, the animals were injected i.p with colchicines 20-22 hr(one cell cycle). 5-Brdu can incorporate into the newly synthesized DNA of replication cells during the S phase of the cell cycle.Bone marrow cells were harvest 2 hr later,

metaphases preparations were made and air dried slides were stored in the dark. For differential SCEs, the slides stained by the Fluorescence plus Giemsa techniques with the use of black light fluorescent lamp according to the protocol of Goto et al. (1978). The slides were stained for 20 min in a 0.05% Hoechst 33258 solution, rinsed with distilled water and placed under a black light fluorescent lamp for 45min, covered with McIlavine buffer(citrate phosphate buffer) and stained with 3% Giemsa solution in phosphate buffer (pH 6.8). Only euploid cells (2n=40) with well spread chromosomes were selected and 40 metaphase per each animal were examined microscopically for analysis the frequency of SCEs.The examination was performed under 2500 Xmagnification (25 x eyepieces X 100 objective lens)by a light microscope (Litz, Germany).

Chromosomal aberration assay

Mice were i.p injected with colchicine 2-3h before killing to arrest the cells to metaphase and facilitate visualization of chromosomes. Chromosomal preparations from testes were made according to the technique developed by Evans et al. (1964), and 100 well–spread diakinesis metaphase I cells were analyzed per animal to assess aberrations in five mice/group. Scoring was performed under 2500 X magnification with a light microscope (Litz, Germany).

Statistical analysis

Statistical analysis was performed using SPSS for Windows (Version 20). Data were compared by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test for multiple comparisons. Statistical significance was set at $p<0.05$. The suppression of mutagenicity was calculated according to

$$\text{Suppression}(\%) = \left[\frac{\%\ \text{aberrant cells in group A} - \%\ \text{aberrant cells in group B}}{\%\ \text{aberrant cells in group A}}\right] \times 100$$

Diab& Hassan (2011) using the following formula:

A: represents groups treated with hydrocortisone alone

B: represents groups treated with hydrocortisone + MEGT

Results and Discussion

The frequency of SCEs induced after i.p. treatment with different doses of hydrocortisone is summarized in Table (1). All the tested doses of hydrocortisone (26, 39 and 52 mg/kg b.w) induced a statistically significant increase in the frequency of SCEs with dose-dependent manner. It reached its maximum 9.33±0.72/cell after treatment with the highest tested dose of hydrocortisone compared with 4.48±0.39 for negative control. However, such increase was not reached the value of the positive control group 31.86±1.24/cell. Single, double and triple SCEs were observed in all treated groups. However, quadruple SCEs recoded only in the positive group.

Table 1. Frequency of sister chromatid exchanges (SCE's) induced in mouse bone marrow cells treated with hydrocortisone and MEGT

Treatment and doses (mg/kg b.wt)	% of SCEs/ chromosomes [A]				SCEs/ cells [B]		Suppression rate (%)/ cells
	Single %	Double %	Triple %	Quadruple %	No.	Mean ± S. E.	
I- Negative control	9.44	0.88	—	—	560	4.48 ±0.39[a]	—
II-Positive control (CP)	26.60	10.28	6.94	2.92	3983	31.86±1.24[h]	—
III -Single treatment							
1-Hydrocortisone alone							
26	14.02	1.98	0.10	—	914	7.31±0.73 [d,e]	—
39	15.54	2.22	0.16	—	1023	8.18±0.54 [e,f]	—
52	17.28	2.60	0.28	—	1166	9.33±0.72[g]	—
2-Negative Extract (MEGT) alone	11.46	0.28	—	—	601	4.81±0.51[a]	—
3-Hydrocortisone +MEGT							
26	11.10	0.76	0.08	—	643	5.14±0.64[b]	29.68
39	11.96	1.02	0.10	—	715	5.72±0.57[b,c]	30.07
52	13.22	1.34	0.06	—	804	6.43±0.90[d]	31.08

A:1000 chromosomes analyzed per each mouse and 5000 chromosomes per group. B: 40 metaphases analyzed per mouse and 200 per group (5 mice). Values within the same column follow by different superscript letters are significantly different from one another ($p<0.05$)

These results are in agreement with the previous findings of other authors who reported that "hydrocortisone" is able to induce SCEs in cultured human lymphocytes(Ahmad& Afzal, 2004; Ahmad et al., 2002).Certain related synthetic steroids "dexamethasone" enhanced SCEs frequency in cultured human lymphocytes and in mouse bone marrow cells (Singh et al., 1994). In addition, "estradiol-17β" was reported to induce SCEs and CAs in human lymphocytes (Ahmad et al., 2000). Oral contraceptives,synthetic steroids hormones, induced significant increase in the number of lymphocytes with DNA migration in alkaline comet assay and frequency of SCEs per metaphase as compared with their age matched untreated controls (Biri et al., 2002).

Table (2) summarized the effect of different doses of hydrocortisone on CAs in mouse spermatocytes. The results showed that hydrocortisone induced a statistically significant percentage of CAs in mouse spermatocytes at the tested doseswith the exception of the dose 26 mg/kg b.w in single treatment regimen. This percentage increased with increasing the dose and with longer duration of treatment. The maximum percentage of aberrations reached 10.60±0.75 which represents about 2.5-fold increases compared with the negative control 4.20 ± 0.37. These finding suggested that hydrocortisone caused a destructive effect on genetic material of the germ cells.Earlier studies reported that corticocorticoids exert a number of deleterious effects on the interstitial Leydig cells of the testis, including direct inhibition of testosterone biosynthesis, suppression of Lutenlizing hormone receptor expression, and induction of Leydig cell apoptosis (Monder et al., 1994;Gao et al., 2002).

Referring to, the mechanism of action of hydrocortisone, it was reported that, steroid hormones can enter the cells by simple diffusion through the cell membrane. The specific target cells possess specific receptor proteins in the cytoplasm, which bind with the steroid molecules, and then these steroid-receptor complexes pass through the nuclear membrane to form steroid-receptor protein-DNA complexes and influence gene expression (Rubin, 1982).

With respect to the types of the induced aberrations, it was observed that dissociated univalents dominated (autosomal and X-Y univalents). The process of chromosome pairing and the formation of bivalents during meiosis are necessary for proper genetic recombination and segregation of chromosomes. The absence of bivalents and the formation of unpaired chromosomes at the time of induction of the first anaphase, may lead to random segregation of homologous chromosomes i.e aneuploidy (Sosnowski et al., 2011). The results show that X-Y univalents were more frequent. This type of abnormality has been discussed as an indicator of male sterility (Burgoyne et al., 1992). Azoospermia was observed in 23-years old patient with testicular adrenal rest tumor received hydrocortisone.However, azoospermia and infertility may be reversible by replacing hydrocortisone with short courses of equivalent dosage of dexamethasone(Claahsen-van der Grinten et al., 2007).

Translocations in the form of chain IV were observed in a low frequency. Several studies reported that chromosomal translocations (rings and chains) have been observed in mice after ionizing radiation and rarely after chemicals (Fahmy&Diab, 2009; William& Hsu, 1980). A significant increase in the frequencies of stable translocations in patients several years after the end of chemotherapy was reported by Genescaet al. (1990). Metaphases with aneuploidy (hyperhaploid) were observed in hydrocortisone groupsimplying its aneugenic activity.

In the present work, mice treated with MEGT alone didn't induce any significant difference in the percentage of SCEs and CAs as compared to control suggesting lack of its genotoxic effect. These results are in line with Ogura et al. (2008) who reported that, oral administration of green tea preparation up to 2000mg/kg did no induce significant increase in micronucleated polychromatic erythrocytes in bone marrow cells ICR CD mice and SD rat.

Oral administration of MEGT to hydrocortisone-treated mice significantly lowered the occurrence of SCEs in mouse bone marrow cells (somatic cells) and CAs in spermatocytes (germ cells) compared with the corresponding

Table 2. Effect of hydrocortisone and MEGT on chromosomal aberration in mouse spermatocytes.

Treatment and doses (mg/kg b.wt)	% of metaphases with different types of chromosomal abnormalities							Abnormal metaphases		*Suppression rate (%)*
	X-Y uni	Auto.uni	X-Y + Auto. uni	Break and/ or fragment	Chain IV	Aneup	Poly			
	%	%	%	%	%	%	%	No.	Mean % ± S.E	
I- Negative control	2.40	1.40	—	0.40	—	—	—	21	4.20±0.37[a]	
II-Positive control (CP)	4.00	3.20	0.40	1.20	0.40	—	—	46	9.20±0.37e	
III -Single treatment for 24 hr 1-Hydrocortisone alone										
26	2.40	1.80	0.20	0.40	0.20	0.40	—	27	5.40±0.40[b]	
39	3.20	2.40	—	0.40	—	0.20	—	31	6.20±0.86[c]	
52	2.20	3.80	—	0.60	0.20	0.20	0.40	37	7.40±0.75[d]	
2-Negative Extract (MEGT) alone	3.60	0.80	—	—	—		—	22	4.40±0.81[a]	
3- Hydrocortisone +MEGT										
39	2.60	2.00	—	0.40	—	—	—	25	5.00± 0.32[a,b]	19.35
52	2.60	3.00	0.20	0.40	—	—	—	28	5.60± 0.93b,c	24.32
IV-Repeated treatment for 7 days										
1-Hydrocortisone alone										
26	3.60	2.20	0.20	0.80	—	0.40	—	36	7.20±0.97[d]	——
39	4.00	3.40	—	0.80	0.20	0.60	—	45	9.00±0.82[e]	——
52	3.20	4.40	0.20	1.60	0.20	0.40	0.60	53	10.60±0.75[f]	——
2-Negative Extract (MEGT) alone	3.40	1.40	—	—	—	—	—	24	4.80±0.58[a,b]	——
3- Hydrocortisone +MEGT										
26	3.40	1.60	—	—	—	—	—	25	5.00±0.71[a,b]	22.00
39	3.60	2.40	0.40	0.40	—	—	0.20	35	7.00±0.84[d]	22.22
52	3.00	3.60	—	1.00	—	—	—	38	7.60±0.60[d]	28.30

500 metaphases were examined in 5 mice per each experimental group (100 metaphases/mouse). Uni: univalent, Aneup: Aneuploidy, Poly: polyploidy. Values within the same column follow by different superscript letters are significantly different from one another ($p<0.05$)

mice treated with hydrocortisone alone. Such suppression ranged from 29.68–31.08% in bone marrow cells and from 19.35–28.30% in spermatocytes. According to our results, it shows that MEGT interacted with the hydrocortisone or its active form in a desmutagenic manner. The antimutagenic and anticlastogenic activities of tea polyphenols are mostly due to their antioxidant activity that inactivates direct carcinogens (desmutagenicity). The antioxidant activity of green tea was reported to be greater than vitamins C and E (Wiseman, 1997). Beside acting as scavenger for reactive oxygen and nitrogen species, green tea also enhances the level of some detoxifying enzymes (Sohn et al., 1994).The procarcinogen activating enzyme cytochrome P450 is also suppressed (Muto et al., 2001).Green tea and EGCG also bind to metal ions and further reduce the generation of reactive free radicals (Hider et al., 2001).

Similar results were obtained by Tanaka (2005) who demonstrated that green tea polyphenols EGCG and (+)-catechin decreased the frequencies of SCEs induced by the carcinogens, paraquat which is a generator of reactive oxygen species and nitrogen oxide in cultured mammalian cells. Earlier studies illustrated that green tea catechins could suppress the genotoxic activity of various carcinogens both in *vitro* and in *vivo* systems (Ito et al., 1989; Sasaki et al., 1993; Sinha et al., 2005).

Conclusions

Overall, the results from this study indicate that hydrocortisone is capable of inducing DNA damage in mice as revealed by the two cytogenetic endpoints used. The observed inhibitory activity of MEGT may be attributed to their antioxidant activity. These results suggested that MEGT interacted with the hydrocortisone extracellulary in a desmutagenic manner.

Acknowledgment

This work was supported by Student Research Committee (grant number.11/8/5), National Research Center, Cairo; Egypt. This study is a part from PhD thesis of Kawthar AE Diab.

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