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Repeated dose studies with pure Epigallocatechin-3-gallate demonstrated dose and route dependant hepatotoxicity with associated dyslipidemia

Balaji Ramachandran[a,*], Subramani Jayavelu[a], Kanchan Murhekar[b], Thangarajan Rajkumar[a]

[a] Department of Molecular Oncology, Cancer Institute (W.I.A), No. 38, Sardar Patel Road, Adyar, 600 036 Chennai, India
[b] Department of Oncopathology, Cancer Institute (W.I.A), No. 38, Sardar Patel Road, Adyar, 600 036 Chennai, India

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ABSTRACT

EGCG (Epigallocatechin-3-gallate) is the major active principle catechin found in green tea. Skepticism regarding the safety of consuming EGCG is gaining attention, despite the fact that it is widely being touted for its potential health benefits, including anti-cancer properties. The lack of scientific data on safe dose levels of pure EGCG is of concern, while EGCG has been commonly studied as a component of GTE (Green tea extract) and not as a single active constituent. This study has been carried out to estimate the maximum tolerated non-toxic dose of pure EGCG and to identify the treatment related risk factors. In a fourteen day consecutive treatment, two different administration modalities were compared, offering an improved [i.p (intraperitoneal)] and limited [p.o (oral)] bioavailability. A trend of dose and route dependant hepatotoxicity was observed particularly with i.p treatment and EGCG increased serum lipid profile in parallel to hepatotoxicity. Fourteen day tolerable dose of EGCG was established as 21.1 mg/kg for i.p and 67.8 mg/kg for p.o. We also observed that, EGCG induced effects by both treatment routes are reversible, subsequent to an observation period for further fourteen days after cessation of treatment. It was demonstrated that the severity of EGCG induced toxicity appears to be a function of dose, route of administration and period of treatment.

1. Introduction

With overwhelming literature, consumption of EGCG (Epigallocatechin-3-gallate) containing preparations in the form of GTE (Green tea extract) or purified preparations, has been documented to show a wide variety of beneficial health effects such as prevention of cancer, on obesity, hyperglycemia, dyslipidemia, elevated blood pressure, inflammation, angiogenesis, against cellular oxidation, [68,76,13], against neurological diseases [44,11,57], arthritis [1], hepatoprotective, against testicular toxicity, cardiotoxicity [15,80,12,59,38] and to improve insulin resistance [39]. Due to such numerous favorable health benefits and gaining popularity, EGCG is widely marketed as a nutraceutical supplement.

In spite of this, there is considerable skepticism regarding the safety of consuming EGCG. Toxicity studies conducted using GTE or purified preparations are reported to exhibit potential toxicities at high doses [4,65], that can provoke nephrotoxicity, aggravated colitis and colon carcinogenesis, down-regulated expressions of anti-oxidant enzymes and molecular chaperones. Low and medium doses of GT polyphenols ameliorated colitis, suppressed down regulation of self defense proteins [35,21,48,17]. Consumption of EGCG containing preparations during pregnancy could increase fetal leukemia risk [35]. Hepatitis and cholestasis are reported as the most serious adverse effects due to continuous consumption of huge quantities of green tea or GTE or EGCG capsules, as evidenced by *in vitro*, *in vivo* and clinical reports [29,54,78,32,18]. EGCG consumption could increase intracellular reactive oxygen species and the effects of EGCG upon cellular oxidation is still uncertain and was argued both as an anti-oxidant and pro-oxidant [27,81,20]. EGCG showed strong anti-oxidant activities *in vitro*. However, such effects are not consistent and cannot be demonstrated *in vivo*. EGCG can

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; GTE, green tea extract; i.v, intravenous; i.p, intraperitoneal; p.o, oral.

* Corresponding author.
E-mail address: balajiphd@gmail.com (B. Ramachandran).

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induce oxidative stress [35,70] and therefore, can cause adverse effects, due to its pro-oxidant activity [43]. Despite such adverse effects, the majority of pharmacological actions of EGCG rely on its pro-oxidant activity such as, to kill cancer cells by forming H_2O_2 [31].

On the other hand, few studies express minimal or no concern; over the genotoxic, teratogenic, reproductive, dermal, acute and short term toxic potential of GTE [49,22–24]. More often, the toxicity of EGCG was studied as a component of GTE or purified preparations [71,3,19,6,22–24]. Studies with extracts that have been extracted with standard extraction procedures, show almost comparable or varied toxicity profile, for *e.g.*, NOAEL's (no observed adverse effect level) for two 90 day oral toxicity studies of GTE in rats and mice, were 500 mg/kg [3,24], 764, 820 mg/kg/day [67], while few 28 day oral studies reported a NOAEL of 2000 mg/kg [6], 2500 mg/kg/day [19], regardless of slightly varying percentage of total EGCG content. Hot water, methanolic, hexane, phenolic and non-phenolic GT fractions in doses of 500 mg/kg to 2500 mg/kg did not cause acute hepatotoxicity [61]. Nevertheless, it is apparent that biological effects in the form of NOAEL's could vary depending upon the variety of source plant material chosen, the method of extraction, on the purity and concentration of the active principle component EGCG and its associated polyphenols being extracted, duration of the study and hence, cannot represent a reproducible toxicity scenario [24,72,73,75,19,3,67,47].

Hence, an appropriate characterization of limiting dose, bioavailability, safe route of exposure, duration of treatment and the nature of toxicity of the pure active principle EGCG as a single constituent, may allow a better understanding of the potential side effects and choosing a dose and dosing frequency that is within the safety window. Hence, we designed a repeated dose maximum tolerated dose study to assess the dose and route dependant toxicity and other potential treatment related effects with pure EGCG (>98%) ranging from high doses to acceptable intake levels, in two administration modalities in adult female swiss albino mice.

2. Materials and methods

2.1. Chemicals

EGCG was purchased from Cayman chemical company, USA, DMSO (Dimethyl sulfoxide) from MP Biomedicals, California, USA and ketamine from Aneket, Neon Laboratories Limited, Palghar (Thane), India. Ortho-phosphoric acid (85%), methanol, ethyl acetate and acetonitrile (HPLC grade) were purchased from Merck.

2.2. Experimental animals

Healthy adult female swiss albino mice (around 6 weeks) were purchased from King Institute of Preventive Medicine, Chennai, India. Animal colonies were housed at the departmental animal facility in clean polypropylene cages and were fed *ad libitum*, with laboratory rodent diet (Nutrilab® Rodent-IR, Provimi, India). Throughout the study, animals had free access to fresh potable drinking water filtered through the aquaguard water filtration system *via* feeding bottle. Animals were acclimatised for a period of two weeks before experimentation. Care of animals complied, according to the regulations of CPCSEA (Committee for the purpose of Control and Supervision of Experiments on Animals), Ministry of Environment, Forest and Climate Change, Government of India. All animal studies were carried out with prior approval from the Institutional Animal Ethics Committee of Cancer Institute (W.I.A), Adyar, Chennai. The animal room was maintained within a temperature range of 22–25 °C and a relative humidity of 50 ± 10%. There was a cycle of 12 h light/dark (lights on at 06:00 AM).

2.3. Study design

Dosage was chosen taking into account, the pubchem available LD_{50} for EGCG as 2170 mg/kg. One tenth of LD_{50} was chosen as starting higher dose for repeated dose toxicity study; with a default dose progression factor of 3.2 that corresponds to a dose progression of one half log unit. Nulliparous, swiss albino mice (around 8 weeks) were randomised into various experimental groups based on body weight, in all the studies. EGCG was dissolved in 4% DMSO diluted in saline as vehicle, irrespective of treatment routes. Various concentrations of EGCG were freshly prepared, immediately before the treatment. Control group received mock treatment with vehicle alone.

Experiment no.1

Animals were grouped into the following experimental groups (n = 5 per group); control (0), 217, 67.8, 21.1 and 6.6 mg/kg/day and dosed (100 μl volume) through p.o (oral) route of administration with the aid of gavage needle, for 14 consecutive days followed by 14 days of observation without treatment (total of 28 day study).

Experiment no.2

Animals were grouped into (n = 5 per group); control (0), 108, 67.8, 21.1 and 6.6 mg/kg/day and dosed (100 μl volume) either through p.o or i.p (intraperitoneal) route of administration, with the aid of disposable 26 G syringe with needle, for 14 consecutive days followed by immediate sacrifice after 24 h of the last dose (total of 14 day study).

Experiment no.3

Animals were grouped into (n = 5 per group); control (0), 67.8, 21.1 and 6.6 mg/kg/day and dosed (100 μl volume) through i.p route of administration, for 14 consecutive days followed by 14 days of observation without treatment (total of 28 day study).

Whole animal body weight was measured twice a week throughout the experiment and the data is expressed as% body weight change during treatment, in comparison to day 1 body weight of the same animal, just before the initiation of treatment. At the end of each study, the animals were fasted overnight, anesthetized with ketamine (78 mg/kg, i.p) and sacrificed by cervical dislocation. Whole blood and serum were collected by cardiac puncture for hematological and biochemical analysis, respectively. Immediately after sacrifice, the animals underwent necropsy. A gross anatomo-pathological investigation was carried out before organ excision. The following organs/tissues have been sampled for histopathological examination and fixed in 10% neutral buffered formalin: liver, kidney, stomach, small and large intestines, brain, spinal cord, spleen, heart, thymus, lungs, trachea, uterus, ovary and bone marrow. Formalin fixed tissues were paraffin embedded, sectioned and stained with haematoxylin and eosin.

2.4. Analysis of complete blood count

Whole blood samples were collected separately in tubes containing EDTA, for the determination of complete haemogram. Hb (haemoglobin), PCV (packed cell volume), TCRBC (total count of red blood corpuscles), TCWBC (total count of white blood cells), DC (differential count), platelets, MCH (mean corpuscular haemoglobin), MCV (mean corpuscular volume) and MCHC (mean corpuscular haemoglobin concentration) were analyzed with haematology auto analyzer MICROS-60, France, as per the manufacturer's instructions.