Effect of green tea on hepatocytes

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Abstract- Tea is one of the most commonly consumed beverages across the world. Green tea, among all the available varieties of tea, has come under the spotlight in last decade. It is mainly because of the plethora of advantages it has for the body. It is rich in catechins, polyphenols and amino acids. These chemicals have a lot of positive effect on the body; from helping in weight loss to reducing the risk of cancer. Green tea has shown curing properties whereby they have successfully reversed the damage done by harmful chemicals such as pesticides. But dose of green tea is crucial. Green tea consumed in larger or more than optimum amount has shown damage to the liver and nasal passages. The various catechins present are (+)-catechin, (-)-epicatechin, (+)-gallocatechin, (-)-epigallocatechin, (-)-epicatechin gallate, (-)-epigallocatechin gallate, (+)-gallocatechin gallate. Of all these chemicals, scientists have found Epigallocatechin gallate(EGCG) to cause the most damage. Decrease in body weight along with reduction in the weights of individual organs, were observed. The blood plasma and the liver were mainly studied to look for the effects of GTE..

Keywords- Green Tea, hepatocytes, EGCG, catechins, liver, blood plasma, body weight, tissues.

1. Introduction:

Tea is the second most widely consumed beverage after water. There are mainly three types of tea available-black, oolong and green tea. They all are collected from the same plant, *Camellia sinensis*. The difference comes because of the different treatment that the leaves are subjected to. Green tea has become quite popular among the masses due to its proven advantages. Over the years, scientists have proven that green tea has a lot of medical benefits. It has been proven to cure a lot of diseases. It has a lot of bioactive compounds which helps in reducing inflammation as well as fight cancer, alongside improving brain function, burning fat. Green tea has a large number of antioxidants which have been proven to prevent a variety of cancers such as breast cancer. Green tea extract is also known to show rejuvenating effects. It has shown to reverse the damage caused by various harmful chemicals on human tissues. Initially, the tissues or cell lines are treated with the chemicals for some stretch of time, enough to cause damage to them. Once some damage is observed, they are treated with green tea extract.

Now, the tissues or cell lines can either be treated with green tea extracts just by itself or in combination with other nutrients and proteins for better effector just with the components isolated from green tea extracts, such as epigallocatechin-3-gallate.

In 2013, Kitadate et al. published a paper studying the effect of oligonol in inducing nasal toxicity in Crl:CD (SD) rats over a time period of 13 weeks. The doses of oligonol, 100, 300, 1000 mg/kg/d, which were injected into the rats were not enough to induce any kind of nasal toxicity. This was observed after a histopathological analysis of the nasal cavity tissue.

Chan et al. researched the effects of green tea extracts on male and female F344/NTac rats and B6C3F1 mice. The research was carried on for 14 weeks and a maximum of 1000 mg/kg of GTE was given to the male and female rats and mice. Necrotic liver accompanied with changes to nose, mesenteric lymph nodes and thymus. The mice showed changes in Peyer's patches, spleen and mandibular lymph nodes.

The reduction in food intake can have a worse effect on the condition of mice and rats which were used in the testing. Peters et al. found that administration of GTE doses while the rats and mice were in empty stomach seemed to cause a spike in the observed toxic levels. They reduced the food intake by gradually decreasing the food while administering 185mg/kg of caffeine orally over a course of 14 days.

Boyd et al. administered albino female rats with caffeine over a period of 100 days to check their toxicity in the intragastric cannula. The maximal dose of $LD_{0(0.1L)}$=110±2.5mg/kg did not kill any of the mice but were observed to cause multiple effects on the mice such as mild cerebral hyperemia, occasional psychotic like self mutilation, so on and so forth.

2. Materials and methods

I. CHEMICAL COMPOSITION OF GREEN TEA:

Green tea is a widely consumed form of tea, made from the leaves and buds of *Camelia sinensis*. It is found to be rich in a variety of chemical compounds. A group of polyphenols, known as catechins constitute about 30% of the dry leaf weight. The types of catechins found in green tea extracts are: (+)-catechin (C), (−)-epicatechin (EC), (+)-gallocatechin (GC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate

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(ECg), (−)-epigallocatechin gallate (EGCg), and (+)-gallocatechin gallate (GCg). A variety of techniques have been used by various scientists to record the chemical compounds of green tea and quantify them. Mass spectrometry has often been relied upon for a comprehensive detection of small metabolites. It has been put to use by being couples with a variety of other techniques to identify or quantify the amount of chemical compounds in green tea. It was mixed Liquid chromatography**[9][14][15][16][20][39].** Capillary Electrophoresis is another oft used technique. **[13][14][15].** However, LC is the one that is coupled with a various other techniques to carry out the identification and quantification process. High performance liquid chromatography was used to analyse individual catechins and caffeine from green tea, simultaneously**[21].**

HPLC was combined with UV-Vis to verify the compatibility of the performance with required performance routine analysis of the phenolic compounds and caffeines from the various teas**[2]**. UPLC(Ultra performance liquid chromatography) was coupled with Mass Spectrometry(MS), Diode Array Detector(DAD) and chemometrics analysis to analyse 68 compounds and identify 54 of them from three different types of teas, based off retention times, UV spectra and MS spectra**[51].** Principal Component Analysis(PCA) was an important method to differentiate between tea samples. Catechins from tissues were identified by Liquid Chromatography Luminescence catechins are released from conjugation by using ascorbic acid during homogenisation process and digestive enzymes. The homogenate is precipitated using acetonitrile or ethanol and ethyl acetate was used for extraction. Analysis was done by HPLC coupled with chemiluminesence or coulometric array detection**[12][11].** Separation of the components of green tea was also carried out by Capillary Electrophoresis detection**[3].** CE is a comparatively faster method. The selectivity and sensitivity of the catechins and epicatechins were increased by using fluorescence detectors. Fluorescence and UV detectors were coupled together to detect catechin in human plasma**[7].** Reversed phase LC and UV detection, together helped in identification of catechins from green tea.**[12].** Notably this method was used to identify many catechins compounds. Caffeine identification was also carried out by using water acetonitrile phosphoric acid solvent system with two step linear gradients of acetonitrile concentration. Over time, the method of HPLC was modified to increase the efficiency of the process of identification and quantification, for example, HPLC coupled with UV method**[33][34]** or with PDA detector which helped in simultaneous identification of catechins.

Interpretation: LC-UV detection was a simple, fast and high method to detect 8 catechins simultaneously. The combination of HPLC and PDA detectors was a fast and simple way. UV detection coupled with HPLC proved that endcapped deactivated, monomeric C_{18} columns were preferred over columns which are non-deactivated monomeric or polymeric.

II. PREPARATION OF GREEN TEA:

Inorder to prepare green tea extract, the green tea is combined with mineral water and steeped at room temperature. The tea is then strained into a container with a lid and the extract is refrigerated and the leaves are tossed. If green tea powder is used then approximately 10g of the powder is added to 100 ml of boiling water. **[49].** The mixing is done in a shaking incubator for 10 minutes approximately. The mixture was filtered and freeze dried. Sometimes more than one type of green tea powder was used to prepare extractions. The extractions formed could be varied based on certain criterias such as maintaining different water temperatures, extracting the filtered tea at different points of time or making multiple extractions under the same conditions to produce first, second and third extract**[29].** Sometimes researchers use green tea leaves, powder or extract ordered from particular companies**[32][19][8][35].** The companies keep their information regarding the extracts or the green tea beverages secret mainly because most of them are patented.

Interpretation: Method of preparation of Green Tea Extract is selected on the basis of the EGCG and other chemical composition in them. The method which yields the highest content of the chemical compounds is selected. Otherwise, research has suggested that bottled and canned GTEs have very low EGCG content**[50].**

III. INJECTION OF GREEN TEA EXTRACT:

The green tea extract was often mixed with saline water of a particular concentration and then injected into the model organism or into a cell line**[19].** The green tea was often administered orally into animals. The dose of the GTE was decided as per the body weight of the animal. At times the GTE was introduced combined with starvation of the animals**[31],** or into cancer patients to check their effect on them[**38**] or into animals, although similar species, but with differences**[37]**. Subcutaneous injection of GTE in combination tap water was employed in some cases to administer the GTE into animal models**[26].** Inorder to treat cell cultures with GTE or GTE containing mixtures, the mixture was added to cell cultures which had been previously incubated in a suitable culture medium,

supplemented with bovine serum. The GTE mixture was added in different concentrations. The cells were then incubated for 24 hours**[43].** The incubation time of the cell lines post treatment with GTE mixtures varied, sometimes continuing till 6 days after which the cell growth was assayed by MTT assay**[36]** or the cell cultures, after the first stage of incubation, were divided into different groups and each group is treated separately with the GTE mixture mixed with the cell growth medium and the GTE mixed into the medium along with some other mixture which was deemed appropriate with regards to the experiment**[42][40].**

Interpretation: The method of introduction of GTE into the organism or its cell line can be decided based on the *in vitro* or *in vivo* method of study opted for. Each of those methods has its own advantages and disadvantages. *In vivo* methods are preferred because they help in learning about the immunogenic responses in living organisms.

IV. HEPATOCYTIC EFFECT:

Green tea extract can have either a positive or a negative effect on liver or kidney or other parts of the body. Green tea is also considered to reverse the adverse effect caused by various chemicals, on the human cells, tissues and organs at large. Insecticide, Fenitrothion(FnH) was orally given to male albino rats, either alone or combined with green tea extract**[18].** Rats are divided into groups with each group being given a different combination of FnH and GTE for 28 days. There was an in vivo treatment of rats with the combinations. Post 28 day treatment, tissues from kidney, liver tissues were dissected and the tissues were fixed in 10% formalin solution for 14 to 18 hours. This step was followed by ethanol treatment and paraffin embedment. 5µm thick paraffin blocks are cut to study the effect on tissues. The levels and nature of reduced glutathione, malondialdehyde, glutathione-S-transferase, Plasma cholinesterase, plasma transaminases(AST and ALT) were determined. Tamoxifen citrate(TAM), a hepatic carcinogen, is another chemical which is fed into albino rats to cause negative effect on liver**[17].** The liver was removed and washed with ice-cold isotonic saline and blotted with filter papers and wrapped with aluminium foil and stored at -80ºC. Liver homogenates, prepared with ice-cold 0.1M potassium phosphate, were used to estimate Glutathione-S-transferase(GST), Glutathione peroxidase(GSP), Catalase(CAT), Superoxide Dismutase(SOD), reduced Glutathione(GSH), thiobarbaturic acid reactive substrate(TBARS). GST was determined by spectrophotometer using 1 chloro 2,4 dinitrobenzene. The change caused in an absorbance due to thioester formation was monitored and the specific activity was expressed in nmol/min/mg protein. GPX activity was expressed as U/mg protein. NADPH oxidation in a reaction with t-butyl hydroperoxide and oxidised glutathione was used to measure GPX activity. Decomposition of H_2O_2 by CAT enzyme is used to measure the CAT activity and expressed in U/mg protein. Nitroblue tetrazolium and phenaine methosulphate were used to measure SOD activity in liver tissue spectrophotometrically at 560nm in U/mg protein. Conversion of 2 nitrobenzoic acid to 2-nitro-S-mercaptobenzoic acid using glutathione was used to measure GSH in nmol/mg. TBARS, in nmol/mg protein were expressed using 1,1,3,3-tetrathoxypropane.

For in-vitro treatment of cell cultures, the cultures were assayed using different techniques. Assay is important to check the cytotoxicity of a material on the cell culture. Assay helps to check if the material releases any kind of toxins into the cell cultures. MTT assay on human heart cancer cells**[42]** or human osteosarcoma cells**[43]** was used to check the effect of nutrient media on them, by determining the mitochondrial succinate dehydrogenase activity of viable cells**.** The nutrient media is a combination of lysine, proline, arginine, ascorbic acid, and epigallocatechin gallate and it was applied to breast cancer cell lines to check it's anti-tumor capacity. The MTT assay is a colorimetric assay. It is based on the ability of viable cells to reduce a soluble tetrazolium salt also known as 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) to a blue formazan crystal as an indication of the mitochondrial succinate dehydrogenase activity of viable cells. Another assay used MTS colorimetric assay. It was used to check the effect of green tea on prostate cancer cell lines LNCaP, PC-3 and DU145**[36].** EGCG proved to be quite effective for inhibiting the cell growth.

Rats were treated with isoflurane and sacrificed the rats by exsanguination from the aortic transection**[19].** Blood samples were collected from the abdominal aorta. The concentrations for various chemical compounds were checked from the serum. The chemicals have proven to have hepatotoxic effect, with the use of biomarkers. Serum and liver samples were collected from male and female rats. The serum collected, was incubated with ß-glucouronidase at 37ºC for 45 min, to check the total amount of EGCG by using HPLC and with an electrochemical detector. Malondialdehyde(MDA) levels were measured and absorbance was measured using spectrophotometer at 400-700nm. MDA was immunohistochemically stained. Sequential sections were checked for IHC analyses. Cell death was observed by TdT mediated dUTP dioxigenin nick end labelling. Sometimes the rats are fed EGCG either once daily or given a single dose**[31].** For single dose experiment blood was collected from anaesthetized animals by cardiac puncture method post 24 to 48 hours of EGCG

dosing, whereas for once daily dosing, blood was collected after the last dose of EGCG after 24 hours. Plasma was isolated. Histopathological and biochemical analysis was done on liver. Plasma ALT was the biochemical marker used. MDA determination was done spectrophotometrically from liver. ELISA was used to check plasma levels of 8 isoprostane. Western blot technique used to check protein expression. They analysed urine to check the EGCG-cysteine conjugate using LC/MS.

INTERPRETATION: The cell metabolic activity is checked. This is done by using the MTT assay. It is a colorimetric assay. The measurement is dependent on NAD(P)H-dependent oxidoreductase enzymes, in the cytosolic compartment of the cell. The flux in the levels of the enzyme is an indication of the cellular metabolic activity, which affects the MTT and tetrazolium dye. Reduction in very little MTT is characteristic of cells with low metabolism. This characteristic is in stark contrast to cells with higher metabolism. MTT and MTS are intracellular methods of reduction of tetrazolium dyes whereas WST-1 is an extracellular method of tetrazolium dye reduction. AST and ALT are enzymes found in the liver. Their levels increase when there is an injury in the liver. The ratio of AST/ALT is calculated closely to recognise the source of the damage. AST is found in both heart and liver to a large extent and in the kidney and muscles to a lesser extent.

3. Result:

I. CHEMICAL COMPOSITION OF GREEN TEA:

Catechins, EC, ECG were separated from grape seed extract**[33][34]** and catechins were separated from human saliva**[47]** by LC-UV. The method was successful in separating 9 catechins simultaneously in under 20 minutes. The mobile phase in the LC-UV contains acid which is crucial for the complete resolution of the catechins and efficient chromatography of the compounds. The combination of HPLC and PDA detector is useful in simultaneous detection of four catechins EGCG, EGC, ECG, EC, gallic acid and caffeine. The detection took place in 20 minutes. The PDA detection occurred at 200 to 400 nm**.[47].** The stability of the tea catechins were measured using RP-HPLC**[48].** Tea catechins were also measured in biscuit matrix. This was done using C_{18} reversed phase column which had a gradient elution system of water and methanol and formic acid and a photodiode array and an UV detector. The detection took place at 275 nm. HPLC was combined with fluorescence to detect catechins in Bulgarian fruits**[46]** and red wine and grapes**[23].**

II. PREPARATION OF GREEN TEA EXTRACT:

The polyphenolic content of green tea extract is what determines the biological effect of the extract. Flavanols and flavonols are the two types of polyphenols present in green tea. Catechin (Flavan-3-ols) is the major polyphenol present in green tea extract. It is colourless and water soluble. And it contributes to the bitterness and astringency of the green tea. Catechins are lost due to prolonged heating. Bagged green tea was discovered to have the highest content of polyphenolic compounds. The content reached a maximum point when the water for its extraction was heated at 100ºC. Although the final extract was discarded due to excessive astringency[**29**]. Astringency is defined as a tactile taste. The tongue feels dry and rough. Chemically it is caused due to a precipitate formation between the polyphenols of the tea and saliva. Another component of green tea extract is methylxanthine, which contributes to the antioxidant properties of green tea. DPPH(2,2-diphenyl-1-pichydrazyl) free radical scavenging is a technique to study the antioxidant activity of the green tea sample**[49].** The value of DPPH free radical scavenging was compared with the reducing power to establish the antioxidant power of the green tea extract. The reducing power is measured by reading the absorbance at 700 nm for 10 minutes. Absorbance is directly related to reducing power, which means that high absorbance means higher reducing power. The storage time of the green tea and the storage temperature also seem to effect the polyphenolic content. 2hours to 4 hours of storage at room temperature showed increase in the total flavonoid content and total non-flavonoid content of the green tea extracts. But a substantial decrease was observed in the total flavonoid content and total non-flavonoid content post 6hours to 24hours of storage at room temperature.

III. INJECTION OF GREEN TEA EXTRACT:

The aim of introduction of a drug into the laboratory animal is that the drug enters the body of the animal. Mice and rats were most commonly used lab animals. However studies were conducted on humans as well. There are various methods of introducing the drug. Treating cell cultures with the GTE mixture to check it's effects on cell lines is an *in vitro* method of carrying out drug administration. Such methods have many advantages such as being cost effective[**27**], effective for study taking into consideration the complex treatment regimes. *In vitro* models help with the study of a larger number of study designs. But an *in vitro* model can never completely replicate the outcome achieved by an *in vivo* model. In an *in vivo* model, drug administration can be divided into three broad types- enteral, parenteral and topical. Enteral administration of drug into the lab animal means direct placement of the drug into the gastro-intestinal tract by

either oral, sublingual, intragastric gavage or rectal method. Parenteral methods include methods other than enteral routes and are considered to be more effective than the enteral method of drug administration because parenteral method produces maximum bioavailability of substances since it passes the first-pass effect of hepatic metabolism. Most commonly it is done subcutaneously or by intra-peritoneal method or intra-muscular methods or intra-venous methods. Topical method of drug administration attributes to the applying of the drug on the skin.

Oral gavage of doses is a common practice observed among the experiments conducted. It is a precise method for delivery of doses and also a faster peak absorption of unstable and unpalatable compounds was observed.

Neurologic and gastrointestinal effects were the results of dose-limiting toxicities which were caffeine related. Also the toxic effect increased if the daily food consumption was reduced by more than 50%[**37**].

IV. HEPATOCYTIC EFFECT:

Green Tea Extract (GTE) has stood to its reputation of having cancer preventative properties. EGCG, present in GTE, showed to result in apoptotic characteristics in the cancerous cell lines which inhibited their growth[**36**]. Lipopolysaccharide(LPS) is one chemical that is known to trigger acute inflammatory responses in the body by initiating the release of inflammatory cytokines in the body. Experiments have suggested that GTE significantly reduces the toxicity caused by LPS[**51**]. *In vitro* cell cultures of cancerous cell lines have undergone apoptosis upon the introduction of EGCG into the cell lines[**45**]. GTE has shown to reverse the negative effects of chemicals in the body of the rats[**18**]. Changes such as congestion, haemorrhage and necrosis, which were caused by harmful chemicals, were reversed once GTE was introduced into the system.

GTE has proven to have a negative effect on hepatic cells. However, the effect took varied times to occur in male and female rats[**19**]. The effect can be estimated from the varying levels in the levels of the various biomarkers in the body. The levels of aspartate aminotransaminase(AST) and alanine aminotransferase(ALT) increased and the levels of alkaline phosphatase(ALP) decreased. That was the general observation however the levels of AST, ALT and ALP varied among male and female rats after 72 hours and 48 hours respectively. Even the EGCG levels varied and their amounts varied between the tissues and serum. A higher level was observed of EGCG was observed in the serum compared to the tissues. Also the levels of Malondialdehyde(MDA) varied in tissues and serum of male and female rats respectively after 72 hours and 48 hours respectively. But like EGCG, the levels of MDA were more in serum compared to tissues. Liver experienced morphological changes such as decreased weight, decreased levels of glycogen in hepatocellular cytoplasm of male and females. Single cell necrosis was observed. Inflammatory reactions were one of the observed results along with hepatocellular mitosis, hypertrophy and bile duct proliferation for 48 to 72 hours. TUNEL and caspase-3-positive hepatocytes observed in perilobular area of males and females. PCNA-positive cells were scattered in the liver of the GTE exposed rats. TG signals were seen in hepatocyte nuclei at 48 and 72 hours and a positive reaction was observed in hepatocellular cytoplasm of all lobular areas. GST-P positive foci were large and scattered throughout the slide and consisted of more than 100 hepatocytes.

Plasma alanine aminotransferase(ALT) is an aminotransferase which is generally found in the liver and also in kidney in smaller amounts and helps in breaking down food in order to produce energy. Higher amounts of ALT is an indication of damaged liver. A single dose of 1500mg/kg of EGCG caused 138 fold increase in ALT levels and a significant reduction in survival rates. ALT is considered as a biomarker for hepatotoxicity. Even a 750mg/kg dose of EGCG showed liver damage[**31**]. The induction of the apoptosis was detected using western blot technique for cleaved caspase-3. Malondialdehyde(MDA) is another biochemical marker for oxidative stress and an increase in it's levels could be observed with two daily doses of 750mg/kg of EGCG. A single dose of 1500 mg/kg of caused EGCG oxidation which could be detected in the urine, collected after 24 hours of treatment with 1500mg/kg of EGCG. MT and γH2AX, protein biomarkers, showed a massive increase in levels after two consecutive doses of 750mg/kg. EGCG showed cytotoxicity and addition of any other chemical did not have any effect on this cytotoxicity[**44**]. This cytotoxic effect seemed to increase more when combined with reduced food intake[**37**]. Experiments have been conducted to determine the lethal dose for the EGCG to cause toxic effects. Mild changes, gastric ulcers and minor changes in the organ water level were observed[**4**]. Caffeine at large showed mitochondrial membrane fragility in heart muscle cells and endotheloid cells post 24 hour treatment with 20mM caffeine[**1**]. But not everytime GTE showed toxic effects. Those observed were mild or moderate and existed as long as the GTE was continued[**38**].

4. DISCUSSION:

Epigallocatchin-3-gallate proves to be one crucial component of green tea. Experiments are conducted among scientists. Often the experiments aim to check the cancer inhibiting claims of green tea but some experiments have found out limiting doses for the consumption of green tea extracts. Doses higher than these limiting doses have proven to cause liver damage among some other mild changes to the body. EGCG has tumor growth inhibiting properties. Thus it has proven to be quite effective in preventing cancer. It is believed that EGCG inhibits some enzyme that is crucial to cancer spread. This enzyme is expected to be androgen dependent since androgen is what causes the prostate cancer and EGCG has proven effective against prostate cancer. Also EGCG can interact with other tumour inducing proteins and thus inhibit their growth and multiplication. This in turn prevents cancer spread in the body. EGCG is also known for its rejuvenating properties. More often than not it has been found to reverse the effects of various chemicals which have caused negative effects to the body.

But over intake of anything has negative impact. Same goes with green tea. Many scientists have conducted experiments where they have checked the effect different amounts of chemicals have on the organs. The dose to be introduced was selected based off previous studies conducted by various scientists or institutions. Also introduction of green tea with other chemicals such as saline or in a mixture caused negative effects.

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