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Effect of Tea Polyphenols on Conversion of Nicotine to Cotinine

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Abstract – Nicotine is one of the major hazardous components in cigarette smoke. Nicotine deals a harmful effect to smokers and passive smokers due to its rapid conversion to various carcinogenic metabolites. Nitrosamine-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is believed to cause lung cancers among the nicotine-derived carcinogens. Recent studies report that NNK synthesis can be inhibited by the metabolism pathway to produce a stable metabolite cotinine from nicotine. Tea polyphenols have been known to contain factors to prevent cancers and to retard progression of cancers. This study aims to correlate tea polyphenol's potential for cancer prevention with an accelerated formation of cotinine. The conversion from nicotine to cotinine in the presence of tea extracts or three polyphenols (Catechin, epicatechin gallate, epigallocatechin gallate) was measured in established cell lines and in *Xenopus* oocytes. Among three lines of cell used, PLC/PRF5 and HEK293 cells showed a fast turnover from nicotine to cotinine while HepG2 cell line showed a marginal difference between groups treated and non-treated with tea polyphenols. When *Xenopus oocytes* were microinjected with nicotine, tea polyphenols appear to accelerate the conversion of nicotine to cotinine. Among the polyphenols tested in this study, (+)-catechin showed the best efficiency overall in accelerating conversion from nicotine to cotinine both in the cell lines and in the oocytes. In summary, the present study indicated that tea polyphenols have a positive effect on conversion of nicotine to cotinine.

Key words ☐ Xenopus oocytes, polyphenols, cigarette smoking, green tea, nicotine, cotinine

INTRODUCE

Cigarette smoke causes major health problems directly to smokers and second-handedly to innocent people by environmental tobacco smoke (Hoffman *et al.*, 1996). Nicotine, the major component in cigarette smoke, is responsible for lung cancer and diseases related to circulatory and gastrointestinal system when converted to toxic metabolites (Brunnermann *et al.*, 1997; Carmella *et al.*, 1997; Hecht, 1999). Due to the drive for tobacco smoking especially in developing countries, the smoking-based respiratory diseases become more prevalent worldwide.

As for mortality caused by tobacco smoking, the rate increases annually by 3.9% worldwide (Wynder and Muscat, 1997). The case of lung cancer grows by 2.9% *per annum.* Many reports support that the smokers are susceptible to the lung cancer twice higher than non-smokers (Gupta *et al.*, 1996). The importance of drugs that alleviates toxicity of nicotine cannot be overemphasized. It, however, takes a long time and costs a significant resource in their development (Lackmann *et al.*, 1999). Under the circumstances, re-discovery of pre-existing functional food or additives are considered as more viable option to cope with the harmful effect of nicotine.

Recent studies show that nitrosamine-4-methylnitrosamino-1-3-pyridyl-1-butanone (NNK), carcinogen derived from nicotine, serves as a cause for lung cancer (Lee *et al.*, 1996; Fujiki *et al.*, 1998). When A/J mice were treated with water-soluble green tea extracts, however, onset for lung cancer was delayed by approximately 45% (Chung, 1999). These results imply that green tea's extracts consist of components suppressing carcinogenicity of NNK among many harmful tobacco-specific metabolites. In addition, tea polyphenols have an excellent effect in removing environment hormones and toxin of all sorts of heavy metal (Malik *et al.*, 2003; Palermo *et al.*, 2003). Traditionally, it is known that green tea protects habitual smokers from danger of nicotine. But there are no scientific and systematic reports about this. The efficacy of green tea against the harmful effect of smoking has been postulated to originate from high vitamin C content in green tea. Indeed, tobacco smoking consumes about 25mg of vitamin C per one tobacco in body (Willers *et al.*, 1995; Witschi *et al.*, 1997). As for vitamin C content, green

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tea is 5-8 times higher than lemon. Alternatively, tea polyphenols are postulated to reduce the harmful effect of nicotine since high amount of polyphenols readily precipitate nicotine following tobacco smoking (Prokopczyk *et al.*, 1997).

Despite many lines of reports, it was hardly known about the mechanism how the tea polyphenols reduce the toxicity of nicotine. This study is to focus on whether the conversion of nicotine to cotinine can be enhanced in the presence of tea polyphenols. Recent reports indicate that the pathway from nicotine to cotinine is safer since cotinine is a stable material and the pathway leading to cotinine may inhibit formation of NNK (Trushin *et al.*, 1998; Schuller *et al.*, 1998; Olincy *et al.*, 1997). This report studied the potential of green tea extracts along with tea polyphenols to assisting nicotine to cotinine conversion in cells. To confirm this fact, we studied whether green tea polyphenols speed up the conversion from nicotine into cotinine. We measured the extent of nicotine to cotinine conversion under three experimental setups: direct mixing *in vitro*, cells culture in the presence of tea polyphenols, and co-injection with nicotine and tea polyphenols into *Xenopus oocytes*.

MATERIALS AND METHODS

Nicotine was purchased from Sigma Chemical (St. Louis, MO, USA). Nicotine stock solution was prepared as 10 mM and stored at 4°C as covered in aluminum foil. One mM nicotine was added into in all experiments. Tea extracts was prepared from dried green tea leaves by adding hot double distilled water (100 g of tea leaf per 500 ml of 95°C water). The tea extracts were filter-sterilized before application into the culture media. Tea polyphenols, (+)-catechin, (-)-epicatechin gallate (ECG), (-)-epigallocatechin gallate (EGCG), were purchased from Sigma Chemical Company.

Gas chromatography (GC), HPLC could have been used since GC and HPLC have a good accuracy; however, it was cumbersome to measure a number of samples. A direct measurement protocol was used to accommodate these requirements (Robert, 1995). In order to study the enhanced conversion of nicotine to cotinine by tea extract was studied by following three schemes: conversion in direct mixing of tea polyphenols and nicotine in vitro, in cultured cells incubated with polyphenols and nicotine, and in *Xenopus oocyte* co-injected with tea polyphenol and nicotine.

Cotinine measurement

The cotinine content was measured according to the Direct Barbiturate Assay (DBA) by Barlow and colleagues (1987). Traditionally, the DBA method was used to measure an equivalent content of nicotine which is very labile in cells. To measure the cotinine content, 200 µl of sample or standard is added in buffer or distilled water. Three duplicates were used per one treatment. In each sample, 100 µl of 4 M sodium acetate (pH 4.7) buffer, 40 µl of 1.5 M KCN, 40 µl of 0.4 M chloramin T and 200 µl of 78 mM barbituric acid are added in acetone as 50/50 (v/v) in turn and is mixed well for 10 seconds. This incubation was done for 15 min at room temperature and terminated by adding 40 µl of 1 M sodium metabolism. The absorbance is measured at 490 nm and quantitated with standardized cotinine content. The cotinine concentration was calculated as follows: $[\text{Cotinine}]=(A_{490}-0.0029)/0.0093$.

Direct mixing

The extent of conversion to cotinine was studied *in vitro* using the mixture of 200 µl of 1 mM nicotine and same volume of green tea extracts and polyphenols. The samples were incubated at 15, 25, or 37°C with shaking. The cotinine content was measured at 0, 10, 20, 30, 60, and 120 minutes after incubation in 1.5 ml tubes. Aliquots of 100 µl were subjected to the DBA protocol.

Cell culture

We used cell line derived from hepatocyte, HepG2 and PLC/PRF5 cell. The HEK293 cell, originating from embryonic kidney, was also treated with tea extracts or polyphenols. Cells were cultured for a week. Once the cells reached 100% confluency, they were divided into aliquots. The aliquot of the cell were cultured up to 100% confluency and further cultured in media containing 1 mM nicotine. Later, the nicotine-treated cells were incubated in the presence of tea polyphenols until various time points (0-120 minutes).

Following treatment with tea extracts and polyphenols during 0, 10, 20, 30, 60, 120 minutes, the cell were washed 3-4 times with PBS. Scraper was used to harvest cells instead of trypsin treatment. Cells were collected by centrifugation, and re-suspended in 100 µl of cold PBS. Sonication was applied to lysis the cell at the highest setting for 30 seconds. Incubation media was also collected and stored on ice until cotinine measurement at 490 nm by DBA procedure. For the reversed setup, cells were also pre-treated with tea polyphenols before addition of nicotine. Cells, then, were washed with PBS, and incubated in the medium containing 1 mM nicotine.

Cotinine metabolism in *Xenopus oocytes* injected with nicotine and tea polyphenols

The cotinine measurement on cell lines, treated with nicotine and tea polyphenols, has potential problems considering a lag period in absorption of nicotine and green tea extracts or polyphenols. Microinjection of nicotine and tea polyphenols was utilized to avoid the lag period problem (Lee *et al.*, 1997). Because of the large size, the oocytes accommodate the injection of large volume of samples and avoid injection of large number of cells. Ten to twenty oocytes were sufficient to obtain samples enough for analysis. In this experiment, *Xenopus oocytes* were injected with the two components. *Xenopus*, known as "South African Clawed Frog" are utilized to study a development phenomenon of deformation in embryo by toxic materials of environment. In addition, *Xenopus oocytes* serve as an excellent system for in vivo translational systems (Lee *et al.*, 1998). Stage IV-V oocytes, 1.0-1.2 mm in diameter, were used in this experiment. After the oocytes were removed from the *Xenopus*, they were equilibriated in OR2 solution at 15 overnight. The *Xenopus oocytes* have resilient protective membrane called vimentin, which impedes injection into the oocytes. The membrane was removed manually with a pair of tweezers when 10 or less oocytes were necessary. Otherwise, the membrane was removed enzymatically by type III collagenase (Sigma Chemical, St. Louis, USA).

Nicotine was injected into the animal hemisphere of the oocytes. The injected oocytes were incubated in OR2 media for 10 minutes. Later, tea polyphenols were injected into the vegetal hemispheres and the oocytes were incubated for 0-120 minutes. At the beginning of incubation, tea polyphenols were injected into the vegetal hemisphere immediately after injection of nicotine into the animal hemisphere. Eppendorf micromanipulator was used to deliver the sample volume of 500 nl per injection. The temperatures used in this experiment were 15, 25, and 32°C. Incubation temperature of 37°C was eliminated since oocytes were easily burst at 37°C. After *Xenopus oocytes* were incubated during various periods, the oocytes were homogenized in a dounce homogenizer containing cold PBS and the lysates were passed through a yellow tip 10 or more times. The lysates were cleared by centrifugation and the supernatant was subjected to the DBA assay as described. In addition, the uninjected oocytes were incubated in OR2 media with nicotine and, later, with tea polyphenols to compare the difference in absorption time for the nicotine and tea polyphenols. Data were normally analyzed on 10 replicates for each observation. The distribution of the means was calculated according to the procedure for the standard error of the mean (SE).

RESULTS

This study investigated the efficacy of tea polyphenols in conversion of nicotine to cotinine that is a safer metabolite among others. Throughout the three categories of experiments, the three polyphenols and tea extracts were shown to positively affect conversion of nicotine to cotinine in experiment using direct mixing method and cell culture in the presence of tea polyphenols, and microinjection of tea polyphenols into Xenopus oocytes. These results indicate that tea polyphenols have a potential for accelerating conversion of nicotine to cotinine.

The efficacy of tea polyphenols against nicotine was studied by the extent of cotinine product. In the direct mixing method (Fig. 1), we measured the cotinine content after direct mixing of polyphenols and incubation for various time period (10-120 minutes). The data from this experiment indicate that nicotine converts to cotinine at a higher rate in the presence of the polyphenols. On the other hand, a relatively slow turnover was evident without the polyphenols. This result implies that the tea polyphenols may have a factor to catalyze nicotine into cotinine *in vitro*.

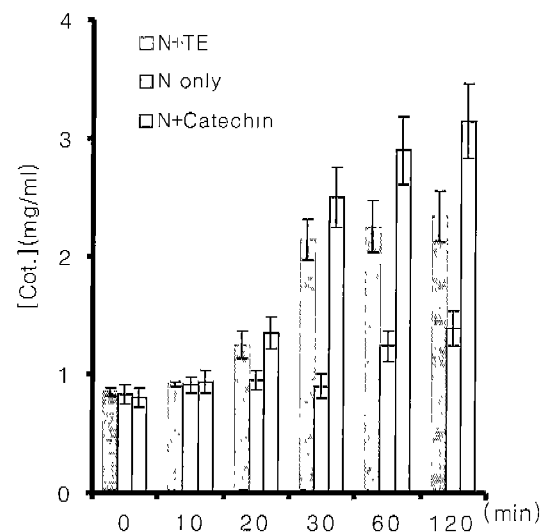

Fig. 1. Nicotine to cotinine conversion in the mixture of tea polyphenols. Cotinine content was directly measured on the mixture of tea polyphenols and nicotine in tubes. At each time point, an aliquot was drawn from the mixture incubated at room temperature. The measurement was made according to the DBA method as described in the Materials and Methods. Error bars represent standard errors (SE). (N: nicotine; GTE: green tea extract; EGCG).

When PLC/PRF5 and HEK293 cells were treated with tea polyphenols, cotinine synthesis appeared to increase for both cell lines. Despite the difference in the origins, the two cell lines showed very similar patterns. Fig. 2-1 represents the typical outcomes of nicotine turnover cells according to cotinine contents measured for HEK293 at the six time points: 0, 10, 20, 30, 60, and 120 minutes. The cotinine measurement increases as the amount of tea polyphenols is raised in the media. The difference in the cotinine content becomes enlarged as the incubation period was prolonged. In terms of cell morphology, however, no significant difference was evident (data not shown). On the other hand, cells were pre-incubated for 6 hours with the tea polyphenols before treatment with nicotine for 0-120 minutes. Fig. 2-2 shows a very similar pattern as in Fig. 2-1. The difference appeared expanding when the cells were pretreated with tea polyphenols. Due to the different experimental setups, a direct comparison may not be appropriate between Fig. 2-1 and 2-2. Based on the difference between each control, the conversion of nicotine to cotinine occurred rather rapidly in the cell pre-treated with tea polyphenols. This result implies that the tea components pre-absorbed in cells are more functional in accelerating cotinine synthesis. In addition, membrane permeability to tea polyphenols is an important factor when the efficacy of tea polyphenol is studied.

Catechin is often mentioned to explain the efficacy of green tea against the toxicity of nicotine. Catechin is known to form precipitants through adsorption with free nicotine; thus, the nicotine can be removed from the solution. According to the increasing amount of cotinine conversion with the increment of tea polyphenols, rather, the efficacy of tea polyphenols against nicotine should be reinforced by a fast conversion of nicotine to cotinine. This result strongly implies that tea polyphenols include a functional component, other than catechin, which can neutralize the toxicity of nicotine by fast converting nicotine to cotinine, a stable metabolite.

Less difference was apparent between treated and non-treated HepG2 cells in comparison to the data from the PLC/PRF5 cells and HEK293 cell (Fig. 3). Although a direct comparison may not be possible between the two cell lines, the different degree of efficacy by tea polyphenol was apparent between the two cell lines. This result may be an indication that the two cell lines present different working environment for tea polyphenol. In converting nicotine to cotinine, the potential intracellular effectors respond to the polyphenols differently depending on the biochemical backgrounds surrounding HepG2 cells.

Cotinine measurement in established cell lines demonstrates inconsistency. This problem may stem from a differential membrane permeability or cell line specific difference in absorbing

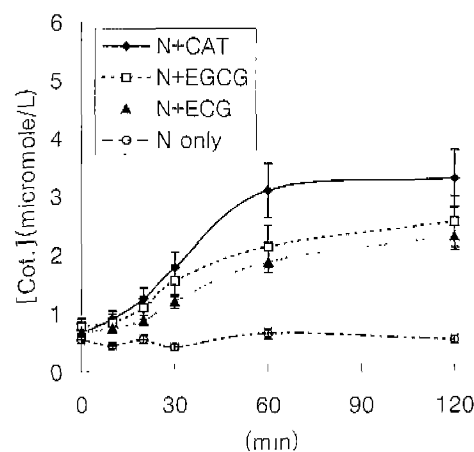

Fig. 2-1. Cotinine measurement on HEK293 cell. Measurement of cotinine was made on the cleared lysates of HEK293 cell. Cells were treated grown with 1 mM nicotine in media for 10 minutes. Later, cells were incubated in the presence of tea polyphenols for the indicated periods. At each time point, the media was collected and measured for the cotinine concentration [Cot.] according to the DBA method. Error bars represent standard errors. (N: nicotine; CAT: catechin; EGCG: epigallocatechin gallate).

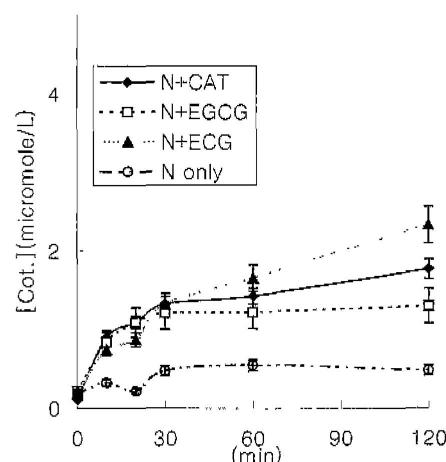

Fig. 2-2. Cotinine formation affected by tea polyphenols pre-absorbed in cells. Cells were incubated in the presence of tea polyphenols for 3 hours before nicotine was added into the media. When the 6 hr pre-incubation was completed, cells were washed copiously with PBS three times and incubated in nicotine containing media. Incubation time in the nicotine media varied as presented in the figure. Cotinine [Cot.] measurement was performed according to the DBA protocols on the cell contents released by sonication as described in Materials and Methods. Error bars represent SE. (N: nicotine; CAT: catechin; ECG: epicatechin gallate; EGCG: epigallocatechin gallate).

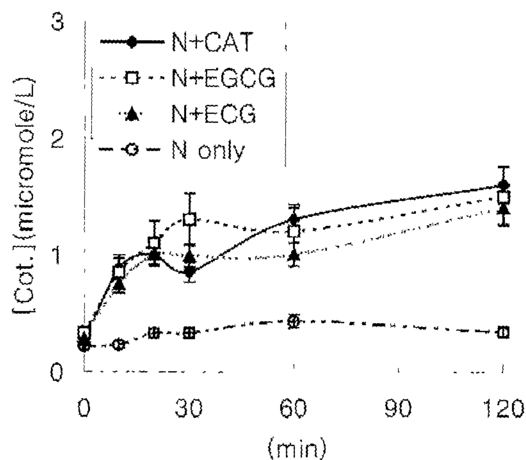

Fig. 3. Cotinine synthesis in HepG2 cells treated with tea polyphenols. HepG2 cells were incubated in nicotine-free media for 7 days up to 100% confluency and incubated in nicotine media for 2 hour until the treatment with tea polyphenols. HepG2 cells were treated with tea extract or tea polyphenols for the periods indicated. Cell lysates were prepared by sonication and cleared by centrifugation at each time point. The cotinine content was measured by the DBA protocol. Error bars represent SE. (N: nicotine; CAT: catechin; ECG: epicatechin gallate; EGCG: epigallocatechin gallate).

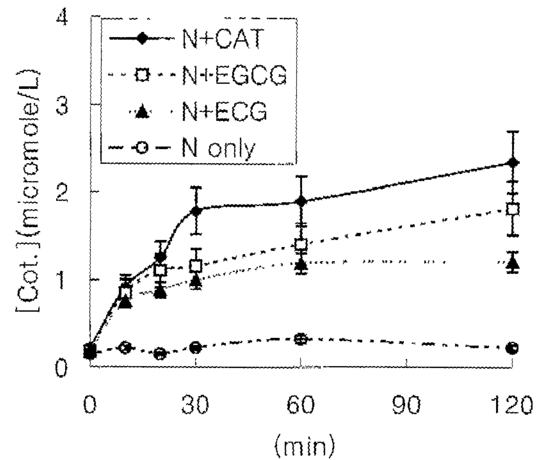

Fig. 4. Cotinine measurement on oocytes microinjected with nicotine. Cotinine measurement in established cell exerts inconsistency among cell lines. This problem most likely stems from a lag period or cell line specific difference in absorbing nicotine from media. Instead, 500 nl of 1 mM nicotine was injected into *Xenopus oocytes*; later, the oocytes were injected with tea polyphenol. Oocytes were incubated up to the time points indicated and homogenized. Cotinine content was measured on the homogenate cleared by centrifugation. Error bars represent standard errors. Error bars represent SE. (N: nicotine; CAT: catechin; ECG: epicatechin gallatc; EGCG: epigallocatechin gallate).

nicotine from media. To circumvent the problems of the differential membrane permeability, a direct injection method was utilized. Oocytes were injected with nicotine or tea polyphenol, separately or in mixture and incubated until various time points.

The cotinine content was measured at the termination of incubation. Fig. 4 shows the results obtained from the injection: The pattern shown in Fig. 4 is similar to that of Fig. 2. When incubation period was gradually extended up to 120 minutes following injection of tea polyphenols, the difference in cotinine synthesis became more distended at each time point compared to PLC/PRF5 cell or the HEK293 cell. This result corresponds with that of direct mixing and cell culture experiments. The direct injection is more effective than the direct mixing method. The injected oocytes were incubated under three different temperatures (15, 25, and 32°C). No significant difference was apparent among the temperatures.

Oocytes were also injected with nicotine and incubated in media containing tea polyphenols. Fig. 5 shows the outcomes of this experiment. Little or no effect of tea polyphenol was shown in this experiment. Taken together with Fig. 4, membrane permeability is a significant factor to assess the function of tea polyphenol. The tea polyphenols seem difficult to pass hrough the oocyte membrane and the cotinine synthesis within

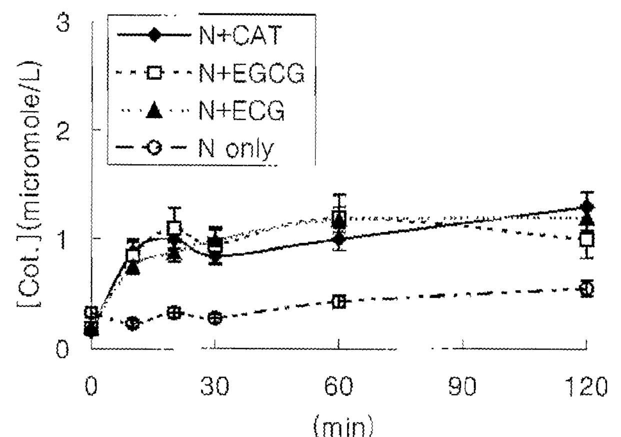

Fig. 5. Incubation of non-injected oocytes with nicotine. To test for the effectiveness of microinjection method, defolliculated oocytes were incubated in OR2 media containing nicotine. The oocytes, then, were washed with fresh OR2 medium and incubated with tea polyphenols in OR2. Cotinine measurement was made on the cleared lysates according to the DBA procedures. Error bars represent SE. (N: nicotine; CAT: catechin; ECG: epicatechin gallate; EGCG: epigallocatechin gallate).

oocyte was not greatly affected in the presence of tea polyphenol in media.

DISCUSSION

Nicotine is the major hazardous component in cigarette smoke. It readily converts to various carcinogenic metabolites such as nitrosamine-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Many scientists believe that NNK arises from the cotinine, a metabolite from nicotine. Recent reports, however, strongly negate that NNK synthesis arises from cotinine. This present study showed that tea polyphenols help breakdown nicotine into cotinine.

Traditionally, the efficacy of tea polyphenol stems from higher content of vitamin C and catechin. Excessive smoking depletes much more vitamin C and green tea, 7-8 times higher than the lemon per unit weight, compensates the loss of vitamin C caused by smoking. Catechin is frequently mentioned as the effective components to precipitate toxic material such as nicotine. Throughout three categories of investigation in this study, tea polyphenol was shown to exert a positive effect on nicotine to cotinine conversion. Despite a discrepancy among established cell lines, cotinine synthesis was greatly enhanced in the presence of tea polyphenols. The enhanced conversion of nicotine to cotinine strongly implies that tea polyphenols mediate the rapid conversion.

The disparity in cotinine synthesis under the presence of different polyphenols among established cell lines indicates that working conditions for tea polyphenol depend on cell lines. The discrepancy can be explained as follows. The presumed effectors, in converting nicotine to cotinine, respond to the various polyphenols depending on the different cellular biochemical backgrounds. Further functional study is necessary to understand intracellular biochemical pathways affected by tea polyphenols. Different cell lines may provide varied stability for the tea polyphenols and even nicotines.

Cotinine synthesis was significantly augmented when cells were pre-treated with tea polyphenols. The pre-absorbed tea component exerts more positive effects in facilitating cotinine synthesis. In addition, membrane permeability to tea polyphenol is an important factor when the efficacy of tea polyphenol is studied. Assuming rapid cotinine synthesis causes a positive effect to the human body, routine tea drinking or tea drinking before smoking may be more effective in nicotine-to-cotinine turnover than after smoking.

The present study might help understand the potential mechanism of green tea in preventing cancer. Our results are potentially significant considering cotinine has been known as the direct precursor to the tobacco-specific lung carcinogen NNK. Cotinine and its metabolites account for 50-60% of nicotine metabolism in humans. Enhanced conversion to cotinine, in the presence of tea polyphenol, may retard the nicotine conversion into the NNK. Among multiple metabolic pathways taken by nicotine, the tea polyphenol may expedite that leading to cotinine which is recently negated as the precursor of NNK. Among many assays in this study, the oocytes showed a definite distinction between groups treated and non-treated with tea polyphenol. This report may be the first report utilizing oocyte injection to measure toxic effect and metabolism. This system could be utilized in many similar studies.

With increasing trend, not only smoking but also damage of indirect smoking could affect the dynamic nature of the developing countries. The more the society develops, the more concerns of field of health and the medical cost increase. Should we develop powder and concentration liquid as additive and know its components, it would be more feasible for us to develop drinks to remove nicotine and highly efficient extraction method of an effective component.

Further characterization may be required for tea polyphenols to be utilized to remove oral nicotine as functional ingredient or to form a best formula in food or food additive. More studies are necessary to prove whether the enhanced conversion to cotinine indeed lower the NNK biosynthesis by diminishing the substrate for NNK. We could offer evidence that tea polyphenol affect the metabolism of nicotine both in mixture and in cells. In conclusion, this present study indicates that nicotine can much better convert to cotinine by tea polyphenols.

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