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Gastrointestinal enhancement of MRI with melanin derived from tea leaves (*Thea sinensis* Linn.)

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Abstract

Melanin was extracted from tea leaves (*Thea sinensis* Linn.) for the first time. Characterization of melanin proved similarity of the original compound to standard melanin. The Langmuir adsorption isotherms for gadolinium (Gd) binding were obtained using melanin. Melanin–Gd preparation demonstrated low acute toxicity. LD_{50} for this preparation was in a range of 1250–1500 mg/kg in mice. Magnetic Resonance Imaging (MRI) properties of melanin itself and melanin–Gd complexes have been estimated. Gd free melanin fractions possess slighter relaxivity compared with its complexes. The relaxivity of lower molecular weight fraction was two times higher than relaxivity of Gd(DTPA) standard. Postcontrast images demonstrate that oral administration of melanin complexes in concentration 0.1 mM provides essential enhancement to longitudinal relaxation times (T_1)-weighted spin echo image. The required contrast and delineation of the stomach wall demonstrated uniform enhancement of MRI with proposed melanin complex.

Keywords: Tea leaves; Melanin; Contrast agent; MRI

1. Introduction

Tea is the oldest folk medicine. It was known in China 5000 years ago due to its stimulating and detoxifying properties (Balentine et al., 1997). The major composition and properties of tea are well documented, but scarce information is available concerning the polymeric polyphenols. Accumulation of polymeric substances is the result of the oxidative coupling of polyphenols occurring during processing of tea leaves (Hara et al., 1995).

Recently, we have disclosed a biopolymer of melanin nature in tea (Sava et al., 2001). Melanin represents a group of black and brown pigments with high molecular weight derived from animal and plant origins. Melanin pigments were regularly extracted from chestnuts, sunflower seeds, black beans (Nicolaus, 1968) and grapes (Zherebin et al., 1982). Unfortunately, natural melanin cannot be produced in sufficient quantities owing to the rarity of extraction sources. The extracted original melanin from tea represents an abundantly available resource with a rather high yield.

Tea melanin could be formed during the growth of tea plant or the subsequent fermentation. The melanin formation in tea is based on the presence of polyphenols and specific enzymes, such as polyphenol-oxidase (Halder et al., 1998) and peroxidase (Digendra et al., 1973). During the fermentation, these enzymes could catalyze the oxidative coupling of polyphenols (Hara et al., 1995).

Melanin was intensively studied for a long time (Nicolaus, 1968; Prota, 1992). The most significant properties concern its chelating capability and high stability under acidic condition (Fogarty et al., 1996).

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Also, melanin possesses paramagnetic properties due to a high concentration of free radicals conjugated to a polymeric matrix. Both paramagnetic and chelating properties render melanin a prospective for Magnetic Resonance Imaging (MRI).

Contrast agents for MRI are in an active research area. A variety of substances tested as contrast agents in MRI have been examined (Unger et al., 1999). The rationale for designing new contrast agents is based on creating complexes with paramagnetic metals (Laniado et al., 1988; Mattery et al., 1987; Rijcken et al., 1994).

Gadolinium (Gd) contained complexes represent the most developed contrast agents (Laniado et al., 1988). However, such complexes are usually not suitable for gastrointestinal investigations. The low pH can cause the dissociation of compounds, particularly serious for Gd(DTPA) and Gd(DTPA–BMA) complexes (Kumar et al., 1993). For example, the half life of Gd(DTPA) at pH 2 is 1.7 h and for Gd(DTPA–BMA), it is 5 min (Tweedle, 1992). Thus, an essential prerequisite for the development of novel oral contrast agents is high stability of Gd complexes in acidic media. Based upon this, melanin becomes the obvious choice (Williams, 1994). Natural melanin also possesses low toxicity, which is beneficial (Nicolaus, 1968). The current study is based on the hypothesis that the paramagnetic and chelating properties of melanin is able to provide a platform for developing stronger and safer MRI contrast agents.

2. Materials and methods

2.1. Materials

In our experiments we used fully fermented Chinese black tea (*Thea sinensis* Linn.). The black tea was purchased from local retail shop in Miaoli, Taiwan. It was identified by Nien-Yung Chiu, the Institute of Chinese Pharmaceutical Sciences, China Medical College. A voucher specimen (GSH-001) was deposited at the Herbarium of this Institute. Gadolinium chloride ($GdCl_3$), Arsenzo III and Sephadex G-50 were purchased from Sigma Chemical Co (St. Louis, MO). All additional reagents were of a chemical reagent grade and purchased from Merck KGaA (Darmstadt, Germany). Commercial preparation of Gd(DTPA) was purchased from Shering AG (Berlin, Germany).

2.2. Extraction and purification of melanin

Extraction and purification of melanin was conducted according to the scheme (Fig. 1) previously designed (Sava et al., 2001) with minor adjustment. Tea leaves were immersed in water at volume ratio 1:10 followed by addition of 10% NH_4OH to adjust pH value to 11. After 36 h incubation, the mixture was filtered and then centrifuged at 20 000 × *g* for 30 min. The extract obtained was acidified by addition of 2 N HCl to adjust pH to 2.5 followed by 2 h incubation at room temperature and centrifugation at 20 000 × *g* for 15 min to pellet melanin. The crude extract obtained was purified by acid hydrolysis, organic solvent (chloroform, ethyl acetate and ethanol) treatment and repeated precipitation. The acid hydrolysis was employed to remove carbohydrates and proteins. Organic solvents were used to remove lipids.

Melanin extracts were hydrolyzed with 7 N HCl at 100 °C for 2 h (Harki et al., 1997) followed by centrifugation at 10 000 × *g* for 10 min, and the precipitate was washed with distilled water. Solid matter was re-dissolved in 1 N NH_4OH and centrifuged at 10 000 × *g* for 10 min. Melanin was precipitated then from the supernatant with 1 N HCl and washed with distilled water. The precipitation procedure was repeated four times followed by a final water wash.

Physical and chemical characteristics of melanin were examined according to typical approaches (Fogarty and Tobin, 1996; Paim et al., 1990; Harki et al., 1997; Prota, 1992; Ellis and Griffith, 1974; Bilinska, 1996; Flip et al., 1974). Infrared (IR) spectra were recorded on Perkin–Elmer spectrometer 1600 FT (Perkin–Elmer Instruments, Norwalk, CT, USA).

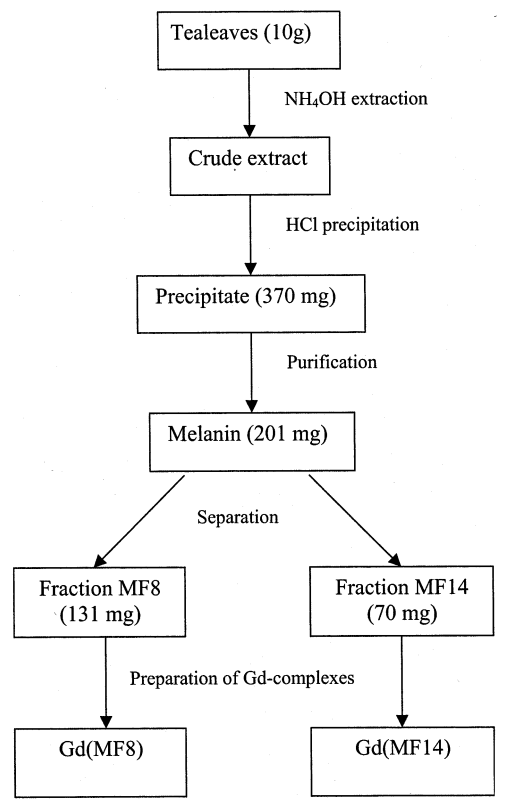

Fig. 1. The procedure of extraction melanin from tealeaves and preparation of melanin–Gd complexes.

Melanin was chromatographed through a Sephadex G-50 in 50 mM phosphate buffer (pH 7.5) at a flow rate of 1 ml/min. Fractions were monitored at 280 nm. The apparent molecular weights (MW) of melanin fractions were estimated with the following size markers: dextran blue (MW 2000 000), aldolase (MW 158 000), bovine serum albumin (MW 66 000), cytochrome C (MW 12 400), and vitamin B12 (MW 1360).

2.3. Preparation of Gd–melanin complexes

Two fractions of melanin MF6 and MF14 with an average molecular weight of 8 and 14 kDa, respectively, were employed for binding of Gd. Both fractions were precipitated with HCl and then washed with water until a pH level of 3–6 was achieved. Each fraction was combined with appropriate amount of Gd^{3+} and then the mixture was stirred for 1 h. Subsequently, complexes were sequestered by centrifugation. Precipitates were washed with distilled water until all traces of Gd disappeared from the wash. Arsenazo III was applied to determine the Gd^{3+} concentration (Rohwer et al., 1995).

The solutions of Gd–melanin complexes were prepared by the following procedure. The precipitates were dissolved in distilled water. The pH was adjusted to 9 by addition of 0.5 N NH_4OH followed by incubation at 50 °C for 1 h. A final pH level of 7.5 was attained due to removal of ammonia by a rotary evaporator under reduced pressure. The end product was filtered through a Nalgene 0.45 μm syringe filter.

2.4. Determination of toxicity (LD_{50})

The experiments were performed using adult Balb/c mice with an average weight of 20–22 g. Animals were housed on a standard rodent chow and water ad libitum. Animals of either sex were distributed into seven groups with an equal number of both sexes in each group comprised ten animals. The experimental groups were treated with the aqueous solution of contrast agent given orally in a dose of 500–1750 mg/kg. The animals were investigated throughout an observation period of 72 h and median lethal dose (LD_{50}) values were calculated by means of probit analysis as described previously (Weber, 1980).

2.5. Measurement of relaxivity (r_1 and r_2) of Gd–melanin complexes

Samples of melanin complexes with Gd in concentrations ranged from 0.05 to 1.0 mmol/l were prepared. Relaxivities were defined in the usual way (Wan et al., 1995). A Bruker Medspec S300 (Bruker Medical GmbH, Germany) at varying, pulse sequences was employed. Namely, longitudinal relaxation times (T_1)-weighted pulse sequences maintained a constant time of echo (TE) at 15 ms while the time of repetition (TR) varied among 200–6000 ms. Furthermore, transverse relaxation time (T_2)-weighted pulse sequences maintained a constant TR at 3500 ms and varied the TE among 40–480 ms. The signal intensity for each of the images was measured. The T_1 and T_2 were calculated for each concentration of contrast agent. Relaxivity r_1 and r_2 values (per mM per s) were obtained by plotting $1/T_1$ and $1/T_2$ as function of concentration followed by a slope determination (Unger et al., 1999). Gd (DTPA) commercial preparation (Schering AG, Berlin, Germany) was used as the standard.

2.6. In vivo MRI investigation

Male rats (Wistar), weighting 320–350 g, were fasted for 36 h, but were allowed drinking water. Prior to the procedure, the rats were anesthetized with a Ketamine/Xylazine (1000/30, w/w). Five ml of Gd(MF8) was orally administered to experimental rats, whereas control rats did not receive it. The contrast agent had a concentration of 0.1 mM. MRI experiments were performed according to standard procedure. The rats were placed in a MRI unit (Bruker Medspec S300) and immediately proton density weighted MRI at a slice thickness of 5 mm were obtained. For T_1-weighted imaging the pulse sequences were $TR = 500$ ms and $TE = 20$ ms and for T_2-weighted images they were $TR = 3000$ ms and $TE = 20$ ms.

2.7. Statistical analysis

All data were expressed as mean ± S.E.M. Differences between groups were considered to be significant at $P < 0.05$ using Student's t-test.

3. Results

According to accepted procedure of melanin extraction (Fig. 1) the average yield of crude product was 3.7%. Purification of melanin gave a 2% yield of pure product.

The amorphous dark-brown pigment extracted from the tea displayed all the physical and chemical properties common to natural melanin (Sava et al., 2001). Furthermore, it was insoluble in both water and organic solvents, such as ethanol, hexane, acetone, benzene and chloroform. As well, it dissolved only in alkali, precipitated in alkaline $FeCl_3$ with pH below 3, bleached in H_2O_2, $KMnO_4$, $K_2Cr_2O_7$ and NaOCl, and produced a blue color in $FeSO_4$/ferricyanide. It showed similar characteristics with standard synthetic melanin (Sigma Chemical Co).

Table 1
Relaxivity of various melanin samples measured in vitro at 20 °C and at 125 MHz

Melanin samples	r_1 (per mmol per s)[a]	r_2 (per mmol per s)[a]
MF8	0.19 ± 0.03	0.79 ± 0.05
MF14	0.23 ± 0.04	0.87 ± 0.06
Gd(MF8)	153.32 ± 10.96	172.1 ± 12.56
	(8.63 ± 0.62)	(9.7 ± 0.71)
Gd(MF14)	134.54 ± 9.52	157.1 ± 13.2
	(6.72 ± 0.48)	(7.85 ± 0.66)

[a] All data calculated using molar concentration of melanin. Data in brackets represent the results obtained calculating Gd molar concentration.

Tea melanin also represented similar to synthetic melanin bonding characteristics in IR spectra. Additionally, IR spectra confirmed the interaction between melanin and Gd. Melanin induced chelating of Gd^{3+} decreased the band of 1720/cm and generated two new bands at 1560 and 1380/cm. It was discovered that there is significant interaction possibility between metal and carboxylic groups at pH 3–6, however, phenolic groups may also be affected. The data obtained are consistent with correspondent results (Paim et al., 1990; Bilinska, 1996).

Melanin thus derived was further purified through Sephadex G-50 column. Two fractions were eluted. Retention volumes of the minor and major peaks were 42.5 ± 2.0 and 61.1 ± 2.0 ml, respectively. The apparent MW for fraction of melanin were 8 ± 2 (MF8) and 14 ± 3 kDa (MF14).

Four varieties of melanin preparations were considered for the relaxivity experiments. MF8 and MF14 were initial fractions of melanin and complexes with Gd were represented by the Gd(MF8) and Gd(MF14) samples. Table 1 illustrates the relaxivities of the mentioned samples. The Gd free samples possess slight relaxivity when compared with its complexes. As it follows from Table 1, melanin–Gd complexes revealed essential increase of relaxivity against melanin itself. Moreover, the lower molecular fraction Gd(MF8) demonstrated greater relaxivity values in comparison with the Gd(MF14) fraction. A 2-fold increase of relaxivity r_1 over the Gd(DTPA) standard ($r_1 = 4.3$/mmol per s) was observed for Gd(MF8).

Fraction Gd(MF8) was chosen for the experiments in vivo due to its higher relaxivity and lower toxicity. Oral median lethal dose (LD_{50}) value for the total number of animals $n = 10$ in each group was estimated. It was found a little difference between LD_{50} of melanin–Gd complexes obtained for MF8 and MF14 fractions. The value of LD_{50} was estimated as 1500 mg/kg body weight for oral administration of Gd(MF8) while acute toxicity of Gd(MF14) was 1250 mg/kg. Oral administration of melanin–Gd complexes to animal in dose of 1250 mg/kg demonstrated mild stimulation of central nervous system. Doses of 1750 mg/kg caused the noticed acceleration of respiration and tremor. Administration of higher doses lead to manifestation of convulsion.

Fig. 2 displays the representative MRI obtained for Gd(MF8) as contrast agent. Fig. 2a and b depict the precontrast and postcontrast images, respectively. Postcontrast images demonstrate that Gd(MF8) provides significant enhancement on T_1-weighted spin echo image. Finally, the required contrast and delineation of the stomach wall revealed that gastrointestinal MRI with the proposed melanin complex was achieved.

4. Discussion

A number of gastrointestinal contrast agents have been developed and tested for application with MRI. As well, there are a variety of contrast agents under development for oral administration. These include the Gd chelates, serving essentially as positive contrast agents on T_1-weighted images (Laniado et al., 1988), as well as superparamagnetic compounds, which act as negative contrast agents on T_2-weighted images. A new contrast agent, developed on the base of natural melanin derived from tea leaves *T. sinensis* Linn., represents the possibility of positive and negative enhancement of MRI.

Weihua reported that melanin could be extracted from the seeds of a tea (*T. sinensis* Linn.) plant (Weihua and Stuugart, 1996). We have been the first to isolate melanin from tealeaves. Characterization of this tea-based melanin indicated that it possesses physical and chemical properties, which made it greatly similar to melanin extracted from alternate sources. However, proposed melanin can be extracted with high yield and in industrial scale quantities.

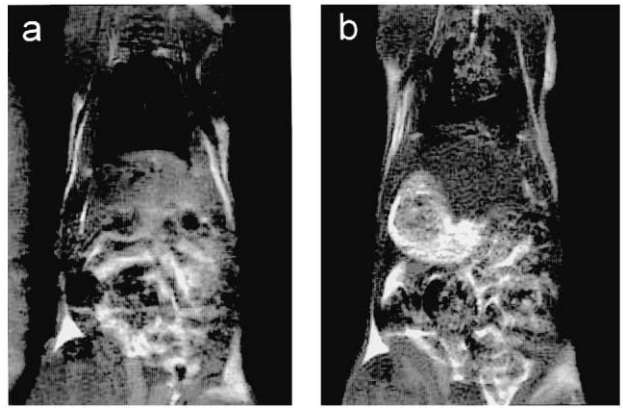

Fig. 2. Precontrast (a) and postcontrast (b) MRI of rat stomach in a 10 min after oral administration of 5 ml 0.1 mM Gd(MF8).

Gd–melanin complexes possess high longitudinal r_1 and transverse r_2 relaxivity, which was found via relaxation measurements (Table 1). Notably, high relaxivity is desirable for several reasons. As the effectiveness of contrast agents is proportional to relaxivity, an agent with much greater relaxivity can be administered in much lower dosages, thus reducing the overall toxicity to which a patient may exposed during MRI examination.

Both Gd(MF8) and Gd(MF14) complexes demonstrated low acute toxicity. LD_{50} for Gd(MF8) was 1500 mg/kg and for Gd(MF14) this value was 1250 mg/kg. Thus, application of melanin complexes for MRI with an effective concentration of 0.1 mM and, correspondingly, in dose of about 12 mg/kg body weight is fully safe.

The batch desorption experiments proved the high stability of Gd–melanin complexes in acidic conditions. In particular, Gd(MF8) fraction released 1.7% of Gd^{3+} during 24 h of equilibration desorption in 0.1 N HCl. This property is very important to use melanin complexes for gastrointestinal investigations.

The proposed complexes demonstrated essential increasing of relaxivity in comparison to the standard Gd(DTPA) contrast agent. Our animal test results indicate that oral administration of melanin–Gd complex provides uniform contrast enhancement.

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