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Vitamin D2 Formation and Bioavailability from *Agaricus bisporus* Button Mushrooms Treated with Ultraviolet Irradiation

SUNDAR RAO KOYYALAMUDI,[†] SANG-CHUL JEONG,[†] CHI-HYUN SONG,[§] KAI YIP CHO,[†] AND GERALD PANG*,[†]

Centre for Plant and Food Science, College of Health and Science, University of Western Sydney, Locked Bag 1797, Penrith South DC, NSW 1797, Australia, and Department of Biotechnology, Daegu University, Gyungsan, Gyungbuk 712-714, Korea

Agaricus bisporus mushrooms contain an abundance of ergosterol, which on exposure to UV irradiation is converted to vitamin D2. The present study evaluated the effects UV-C irradiation on vitamin D2 formation and its bioavailability in rats. Fresh button mushrooms were exposed to UV-C irradiation at mean intensities of 0.403, 0.316, and 0.256 mW/cm^2 from respective distances of 30, 40, and 50 cm for periods ranging from 2.5 to 60 min. Vitamin D2 and ergosterol were measured by HPLC-MS/MS. The stability and retention of vitamin D2 were assessed including the extent of discoloration during storage at 4 °C or at room temperature. Exposure to UV-C irradiation at 0.403 mW/cm^2 intensity from 30 cm distance resulted in a time-dependent increase in vitamin D2 concentrations that was significantly higher than those produced at intensities of 0.316 and 0.256 mW/cm^2 from distances of 40 and 50 cm, respectively. Furthermore, the concentrations of vitamin D2 produced after exposure to UV-C irradiation doses of 0.125 and 0.25 J/cm^2 for, 2.5, 5, and 10 min were 6.6, 15.6, and 23.1 μg/g solids, equivalent to 40.6, 95.4, and 141 μg/serving, respectively. The data showed a high rate of conversion from ergosterol to vitamin D2 at short treatment time, which is required by the mushroom industry. The stability of vitamin D2 remained unchanged during storage at 4 °C and at room temperature over 8 days ($P = 0.36$), indicating no degradation of vitamin D2. By visual assessment or using a chromometer, no significant discoloration of irradiated mushrooms, as measured by the degree of "whiteness", was observed when stored at 4 °C compared to that observed with mushrooms stored at room temperature over an 8 day period ($P < 0.007$). Vitamin D2 was well absorbed and metabolized as evidenced by the serum response of 25-hydroxyvitamin D in rats fed the irradiated mushrooms. Taken together, the data suggest that commercial production of button mushrooms enriched with vitamin D2 for improving consumer health may be practical.

KEYWORDS: ***Agaricus bisporus*; vitamin D2; UV irradiation; bioavailability**

INTRODUCTION

Vitamin D is essential for human health. The functional indicator for dietary reference intake of vitamin D is serum 25-hydroxyvitamin D or 25(OH)D, a metabolite produced in the liver (*1, 2*). There is an association between serum 25(OH)D and a number of preventive and dysfunctional diseases, suggesting that improving vitamin D status may mitigate these disease conditions (*3*). The case for improving vitamin D status in the general population is supported by numerous worldwide surveys (*4*−*7*).

The major source of vitamin D for humans is exposure to sunlight, but this has become a problem due to seasonal shift and latitude, the use of sun screen, and other conditions such as obesity, melanin, and aging, which interfere with solar exposure, leading to vitamin D deficiency (*8*). Vitamin D is plentiful in the food chain (*9*) including oily fish such as cod, salmon, mackerel, tuna, and sardine, but fungus-derived vitamin D is produced by UV-B exposure. Fortification of foods including milk, yogurt, fruit juice, and bread with vitamin D has been promoted as a source of daily vitamin D intake (*9, 10*). Vitamin D supplementation also provides a major source of vitamin D to mainly correct for vitamin D deficiency (*8, 11*). However, in the case of supplementation, it is unclear as to the exact dose required for efficacy additional to that above the "normal" level, which is not accurately defined in the general population, and overdosing could lead to intoxication (*8, 12*).

* Author to whom correspondence should be addressed (telephone +61-2-96859987; fax +61-2-96859915; e-mail g.pang@optusnet.com.au).

[†] University of Western Sydney.

[§] Daegu University.

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Nevertheless, daily dietary intake of natural food with a therapeutic amount of vitamin D may help to improve vitamin D status in the general population rather than one or more foods being fortified with vitamin D.

Several studies have reported the formation of vitamin D, namely vitamin D2, in mushrooms following exposure to UV-B and -C irradiation through conversion of the pro-vitamin ergosterol (*13*−*16*). However, attempts to replicate these studies in a commercial setting have proven to be impracticable for mushroom growers due to the variable amounts of vitamin D content produced, the intensities of exposure, the prolonged exposure time to UV irradiation, the unknown level of retention, and the discoloration of the mushrooms. Thus, the objective of this study is to investigate, first, the conditions required to produce nutritional amounts of vitamin D2 in *Agaricus bisporus*, a white button mushroom popular with consumers worldwide, and, second, to evaluate its bioavailability in vitamin D deficient rats fed UV-irradiated mushrooms.

MATERIALS AND METHODS

Reagents and Equipment. Ergosterol, vitamin D2, 25-hydroxyvitamin D2, and sodium ascorbate were purchased from Sigma-Aldrich Chemicals (Australia). Potassium hydroxide, methanol (HPLC grade), acetonitrile (HPLC grade), *n*-pentane (AR grade), ethanol, anhydrous sodium sulfate (AR grade), and sodium hydroxide were purchased from Lomb Scientific Pty Ltd. (Australia). A Sylvania germicidal UV-C lamp, 30 W, 89 cm length (made in Japan), was used as a source of irradiation. A Minolta CHROMA meter CR-200/CR-210 (Minolta Corp.) was used for measuring coloration. A UVM-CP (Germany) detector was used to measure the UV-C intensity.

UV Exposure. *A. bisporus* mushrooms were supplied from producers on the day of harvest. The mushroom samples were exposed in groups of 10 mushrooms button-side up to UV-C irradiation in a cabinet installed with a UVM-CP detector to monitor UV-C intensity. At 0, 2.5, 5, 10, 20, 30, 40, and 60 min, individual mushrooms were collected at random from each of the nine positions marked in the cabinet. The mushrooms were then pooled and sliced into small pieces before freeze-drying. Three experiments were conducted by exposing mushrooms to UV-C irradiation for various times at distances of 30, 40, and 50 cm from the UV-C source. The irradiation intensities were measured at these distances using a UVM-CP detector. Following irradiation treatment, the mushrooms were collected for analysis of vitamin D2 and ergosterol contents or stored for various times at 4 °C and at room temperature (25 °C) for analysis of retention and stability of vitamin D2.

Extraction Method. Ergosterol and vitamin D2 were extracted as described in other studies (*17, 18*). Briefly, freeze-dried mushroom samples (0.5−1 g) were placed into a 250 mL round-bottom flask and mixed with 4 mL of sodium ascorbate solution (17.5 g of sodium ascorbate in 100 mL of 1 M NaOH), 50 mL of ethanol (95%), and 10 mL of 50% potassium hydroxide. The mixture was saponified under reflux at 80 °C for 1 h and then cooled before transfer into a separating funnel. The mixture was extracted first with 15 mL of deionized water, followed by 15 mL of ethanol, and then three times with 50 mL of *n*-pentane. The organic layers were pooled and washed three times with 50 mL of 3% KOH in 5% ethanol and then finally with deionized water until neutralized. The organic layer was transferred to a rotary evaporator, and the residue obtained was dissolved in a chloroform/methanol mixture (1:3 v/v). The solution was then filtered through 0.45 μm nylon membrane syringe filters for HPLC-MS analysis. Recovery of the extraction process was determined by spiking the samples (n = 3) with known amounts of vitamin D2 and ergosterol. The moisture content was determined in all samples.

Preparation of Standards for Calibration. Standard vitamin D2 (200 μg) and ergosterol (1.0 mg) samples were dissolved in 2 mL of $CHCl_3$/MeOH (1:1 v/v) to give standard solutions containing 100 and 500 μg/mL of vitamin D2 and ergosterol, respectively. For the HPLC-MS/MS analysis, 50, 100, 150, 200, 250, 300, 350, and 400 μL of the standard solution were diluted to 1 mL with methanol to give calibration standards with analyte concentrations ranging from 5 to 40 μg/mL of vitamin D2 and from 25 to 200 μg/mL of ergosterol.

HPLC-MS/MS Analysis. The identification and quantification of ergosterol and vitamin D2 were carried out by high-pressure liquid chromatography with atmospheric pressure chemical ionization mass spectrometry (HPLC-APCI-MS/MS).

The HPLC-MS/MS analysis was performed on a Varian ProStar model 210 gradient solvent delivery module and a Varian ProStar model 430 autosampler with a Varian 1200 L tandem MS/MS detector. Positive atmospheric pressure chemical ionization (APCI) was employed. The autosampler was fitted with a 100 μL loop. Separation was achieved on a Gemini 5 μm C18 column (250 × 2.00 mm) using methanol/acetonitrile (25:75 v/v) as the mobile phase. The sample run time was 30 min. The flow rate was 0.2 mL/min. Different HPLC-MS/MS conditions were determined by direct infusion of individual pure standard of ergosterol and vitamin D2 into the MS detector to determine the settings necessary to obtain the optimum amount of parent and daughter ion(s). The APCI-MS was operated in positive ion mode. The atmospheric pressure ionization (API) housing and drying gas temperatures were maintained at 56 and 350 °C. The electrospray capillary and detector were set 50 and 1800 V, respectively. Argon was used at 2.0 mTorr as the collision gas. For the HPLC-MS/MS analysis, identification was achieved by comparison of retention times of sample and standard. The identity was confirmed by comparison of the observed daughter ions and their relative abundances.

Bioavailability. Sprague−Dawley male rats (100−120 g each) obtained from Daehan Biolink Co., Ltd. (Seoul, Korea), were housed individually in stainless steel cages in a room with controlled temperature (22 ± 2 °C), 55 ± 5% relative humidity, and a 12 h cycle of light and dark. Rats (five per group) were maintained on commercial pellet diet (Sam Yang Co., Korea). This study was approved by the Animal Care and Ethics Committees of the University of Western Sydney, Australia, and the University of Daegu, South Korea. For oral feeding experiments, the freeze-dried mushrooms were ground into a powder, weighed, and then resuspended in saline before use. In each group of eight rats, control rats were administered non-UV-treated mushrooms, whereas the treated groups were administered 50, 100, and 200 mg/kg of body weight (BW) of UV-irradiated mushrooms by oral intubation daily for 3 weeks, respectively. The amount of food intake and the body weight for each animal were recorded daily. Following the final oral dosing, the animals were fasted for 9 h before sacrifice. Plasma samples were collected from heparinized blood by centrifugation at 1100*g* for 10 min. The plasma samples were frozen at −20 °C until analysis.

Quantification of 25-Hydroxyvitamin D2 by HPLC-MS. Plasma samples (1 mL) in borosilicate tubes were precipitated with 200 μL of methanol. After vortexing for 10 s, 1 mL of hexane was added to each tube to extract 25(OH)D2 and then further vortexed for 10 s before centrifugation at 1600*g* for 15 min. The extraction was repeated three times, and the fractions obtained were combined. The solvent was then removed in a rotary evaporator at 50 °C. The residue obtained was reconstituted in methanol for LC-MS analysis.

Each sample separation was achieved using a Varian Microsorb-MV C8 column (150 × 4.6 mm). A linear gradient eluent of A (methanol) and B (0.1% acetic acid in water) was used. The gradient elution was programmed as follows: 0−2 min, 0% A; 2−3 min, 0−70% A; 3−5 min, 70−100% A; 5−24 min, 100% A; 24−26 min, 100−70% A; 26−27 min, 70−0% A; 27−30 min, 0% A. The flow rate was 0.25 mL/min. The mass spectrometer was operated in positive mode with an APCI. The drying gas temperature was set at 350 °C. Capillary, shield, and detector voltages were 50, 600, and 1800 V, respectively. At 50 V of capillary voltage was identified m/z 395.79 $[M + H - H_2O]^+$ product ion for the quantification and identification of 25-hydroxyvitamin D2 (*19*).

Statistical Analysis. The data were expressed as means ± SD. When appropriate, a comparison between treatment groups was analyzed by one-way or repeat measures analysis of variance (ANOVA) or by paired t test. A P value of <0.05 was considered to be significant.

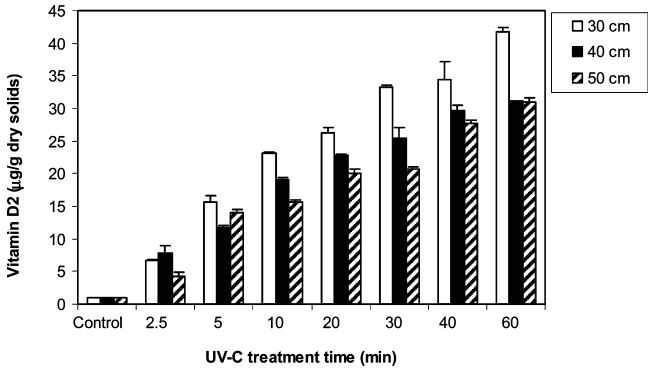

Figure 1. Vitamin D2 concentrations in mushrooms treated with UV-C from three distances at intensities of 0.403, 0.316, and 256 mW/cm^2, respectively. Each value represents the mean ± SD for duplicate samples of pooled mushrooms collected randomly from nine positions exposed to UV-C light. $P = 0.0006$ for comparison between treatment groups with nine mushrooms per group.

RESULTS AND DISCUSSION

HPLC-MS/MS Analysis of Vitamin D2 and Ergosterol. The precursor ions for vitamin D2 and ergosterol are the same (*m*/*z* 397.6, representing $[M + H]^+$), and they produce similar daughter ions at *m*/*z* 271 and 379. Vitamin D2 and ergosterol have the same daughter ions but their retention times were 16.2 and 20.1 min, respectively. The two compounds were well separated. The quantification of vitamin D2 and ergosterol was achieved by extrapolation from a standard curve. The eight-point calibration curves, obtained using a linear fit, used for quantification had r^2 values of 0.9985 and 0.9958 for vitamin D_2 and ergosterol, respectively. The average recoveries of D2 and ergosterol were 83.76 and 86.18% with average RSDs of 3.49 and 9.00%, respectively.

UV-C Exposure. Mushrooms obtained 1 day after harvesting were exposed button-side up to UV-C irradiation at intensities of 0.403, 0.316, and 0.256 mW/cm^2 from distances of 30, 40, and 50 cm, respectively. These distances were chosen to determine whether a strategic placement of a UV-C light in the growers' rooms is effective in triggering the conversion of ergosterol to vitamin D2 in cultivated mushrooms before harvesting. As shown in **Figure 1**, the increase in vitamin D2 concentrations in micrograms per gram of dry solids was dependent on time and intensity of exposure to UV-C irradiation, which in turn was dependent on distance from the UV source. The irradiation dose in joules per square centimter was calculated when appropriate by multiplying the UV intensity by the time of exposure in seconds. Irradiation from a distance of 30 cm at an intensity of 0.403 mW/cm^2 produced higher concentrations of vitamin D2 after treatment for times ranging from 5 to 60 min when compared to those produced with intensities of 0.316 and 0.256 mW/cm^2 at distances of 40 and 50 cm, respectively ($P < 0.0006$). However, from the commercial standpoint, an exposure time of 5−10 min at an UV-C intensity of 0.401 or 0.316 mW/cm^2 from either 30 or 40 cm distance would be considered desirable to produce nutritional levels of vitamin D2. Our results showed that the amounts of vitamin D2 produced after UV-C treatment for 2.5, 5, and 10 min using an intensity of 0.401 mW/cm^2 at doses of 0.063, 0.125, and 0.25 J/cm^2 were 6.7, 15.6, and 23.1 μg/g of dry solids, which, based on local recorded moisture content of 92.7%, were equivalent to 40.6, 95.4, and 141 μg/serving, respectively. As shown in **Figure 1**, even at longer distances (40 and 50 cm) and shorter exposure time to UV-C irradiation (5−10 min), our results showed a greater conversion of ergosterol to vitamin D2 in button mushrooms compared to that reported in the literature, with vitamin D2 concentrations produced ranging from 3.83 to 13.85 μg/g of dry solids using UV-B or -C irradiation (*13*−*16*). However, the conditions used in other studies to effect vitamin D2 formation may not be suitable for application by mushroom growers for a number of reasons. First, the dose and duration of UV irradiation using a combination of irradiation intensities and treatment regimens are too complicated to replicate in the mushroom grower's facility (*13*−*15, 19*). Second, the need for whole body mushroom irradiation including button, gill, and stalk to maximize vitamin D2 formation, the manipulation of moisture content in mushrooms by partial drying prior to UV-B irradiation, and the long exposure time to UV irradiation (up to 2 h) are clearly conditions that would be considered impractical from the industry's point of view (*13, 20*). However, an attempt at standardization of treatment regimen using UV-B irradiation to produce vitamin D2 in button mushrooms for potential application on a commercial scale has been reported (*16*). In that study (*16*), using an intensity of 1.0 mW/cm^2, the concentration of 3.83 μg/g of dry solids or 31.49 μg/serving of mushrooms produced in 8 min is considerably lower than that achieved in the current study with a vitamin D2 concentration of 23.13 μg/g of dry solids or 141.1 μg/serving using an intensity of 0.403 mW/cm^2 UV-C at a dose of 0.25 J/cm^2 and a comparable exposure time of 10 min (**Figure 1**). Thus, our treatment regimen using UV-C irradiation may be more effective and practical for mushroom growers to produce nutritional levels of vitamin D in button mushrooms.

Vitamin D2 Stability. To determine whether vitamin D2 produced in UV-C-treated mushroom is stable, UV-C-treated mushrooms were stored at room temperature or at 4 °C for 8 days. On each day during storage, vitamin D2 concentrations in the mushrooms were measured. Vitamin D2 concentrations remained relatively stable during storage at either 4 °C or room temperature when mushrooms were treated with UV-C irradiation at 0.316 mW/cm^2 intensity at a distance of 40 cm. In terms of vitamin D2 content, storage at either 4 °C or room temperature (25 °C) showed no difference in stability except for an increase in room temperature samples at day 8, which may be due to additional ergosterol conversion to vitamin D2 in mushrooms exposed to the cumulative effects of artificial light during storage. Contrary to the prediction by first-order kinetics that vitamin D2 undergoes degradation during storage over a 5 day period (*16*), our experimental data showed no sign of vitamin D2 degradation during storage for 8 days. Thus, our study with daily sampling of post-UV-C irradiation mushrooms provides a more reliable assessment of vitamin D2 retention/stability than that given by prediction based on first-order kinetics (*16*).

Discoloration. Because exposure to the intensity of UV-C irradiation could lead to discoloration of mushrooms, a time-dependent study was conducted in which mushrooms were treated with UV-C irradiation from distances of 30, 40, and 50 cm followed by storage at room temperature and at 4 °C to determine the effects on color change. There was a subtle visible change in the surface appearance of the mushrooms from whiteness to a brownish tinge with increasing time of exposure to UV-C irradiation. As visual assessment is considered to be unreliable, a more accurate measurement of color change was conducted using a chromometer. As shown in **Figure 2**, a color change from whiteness to blackness on a scale of a 100 expressed as *L* values was measured from 0 to 60 min for mushrooms exposed to UV-C irradiation at a 30 cm distance. There was a gradual decrease in *L* values with color change

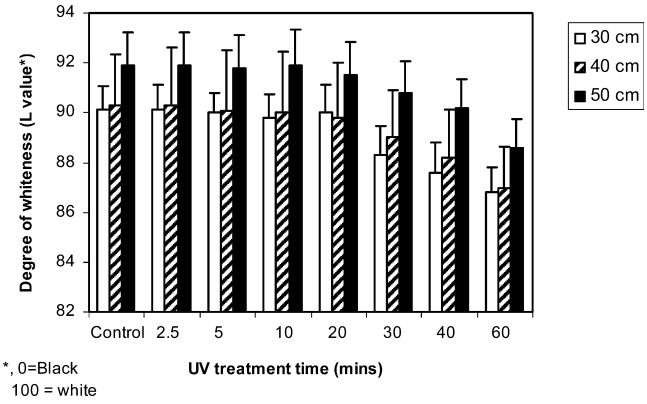

Figure 2. Discoloration of mushrooms exposed to UV-C irradiation from three respective distances at intensities of 0.401, 0.316, and 0.256 mW/cm^2, respectively. Means ± SD represent 10 replicates per group with significant difference between treatment groups ($P < 0.005$).

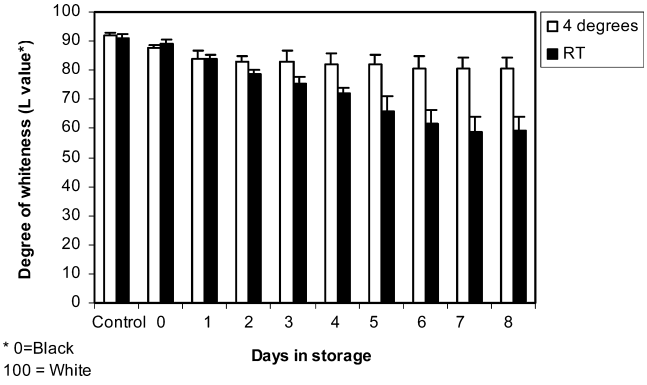

Figure 3. Discoloration of UV-treated mushrooms during storage at 4 °C and room temperature. Means ± SD represent 10 replicates per group with significant difference between treatment groups ($P < 0.007$). Control = non-irradiated mushrooms).

from whiteness to blackness. Similar results were obtained when mushrooms were treated with UV-C irradiation at a 40 cm distance but, as expected, discoloration was least observed when mushrooms were treated with UV-C at 50 cm distance, with a highly significant difference between treatment groups (**Figure 2**) ($P < 0.005$). The color change was more pronounced when the mushrooms were stored at room temperature compared to those stored at 4 °C (**Figure 3**) ($P = 0.007$). Marked color change tended to occur in a time-dependent manner beginning on day 1−2 for mushrooms stored at room temperature (data not shown). In contrast, color did not change significantly in mushrooms stored at 4 °C, indicating that storage of irradiated mushrooms in a cold room may be necessary to maintain quality. Whereas color change in mushrooms as measured by the chromometer was reproducible, an attempt to correlate it with a visual assessment score, especially during the first 2−3 days irrespective of the storage condition, was unsuccessful. Nonetheless, our results are consistent with those reporting that discoloration of mushrooms occurred with increasing UV irradiation time, which is considered to be undesirable from a commercial viewpoint (*13, 15*). Our results demonstrated that a distance between 30 and 50 cm and a short exposure time (<15 min) were proper conditions for mushroom treatment with UV-C irradiation to avoid discoloration of the mushrooms.

Bioavailability. The absorption of vitamin D2 was determined by measuring 25-hydroxyvitamin D2 response in the blood plasma of rats following oral dosing of UV-treated mushrooms. Using HPLC-MS analysis, the calibration curve for quantitation of 25-hydroxyvitamin D2 had a linear fit of r^2 = 0.9984. As shown in **Figure 4**, control rats fed UV-untreated mushrooms for 3 weeks had trace to undetectable levels of plasma 25-hydroxyvitamin D2 compared to rats fed the UV-irradiated mushrooms ($P < 0.0001$). Furthermore, the amount of 25-hydroxyvitamin D2 detected in plasma was dose-dependent, with doses ranging from 50 to 200 mg/kg of BW/day administered daily for 3 weeks. The vitamin D2 concentration per gram of dry solids of irradiated mushrooms was 17.6 μg. As low as 0.88 μg of vitamin D2 per 50 mg/kg of BW/day of freeze-dried irradiated mushrooms was effective in elevating a serum concentration of 3.13 nmol/L of 25-hydroxyvitamin D2. Bioavailability studies in animals and humans taking irradiated mushrooms have also shown an increase in serum 25-hydroxyvitamin D2 compared with controls (*21, 22*). Because 25-hydroxyvitamin D2 is considered to be a marker of vitamin D bioavailability, our data suggest that vitamin D2 in mushrooms is absorbed following oral ingestion. As reported by other investigators, the use of radioimmunoassay to measure serum vitamin D response does not discriminate between 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 (*21*); thus, the bioavailability of vitamin D2 under this circumstance may be overestimated (*21*). Using HPLC-MS/MS analysis, our serum 25-hydroxyvitamin D2 levels are considerably lower than those reported in rats fed UV-irradiated shiitake mushrooms, which gave a serum 25-hydroxyvitamin D2 concentration of 129 nmol/L (*21*). The difference in response may be due to our use of ground dried mushroom solids instead of the homogenized powder, the short feeding time (3 weeks), and the sole detection of 25-hydroxyvitamin D2 by HPLC-MS/MS as opposed to using radioimmunoassay, which detects both vitamins D2 and D3 (*21*). Nonetheless, our results showed for the first time that vitamin D2 from irradiated *A. bisporus* button mushrooms was well-absorbed and metabolized when fed to rats. Whether vitamin D2 from fresh non-freeze-dried UV-treated mushrooms is similarly absorbed and metabolized remains to be determined, but such study can be conveniently carried out in humans.

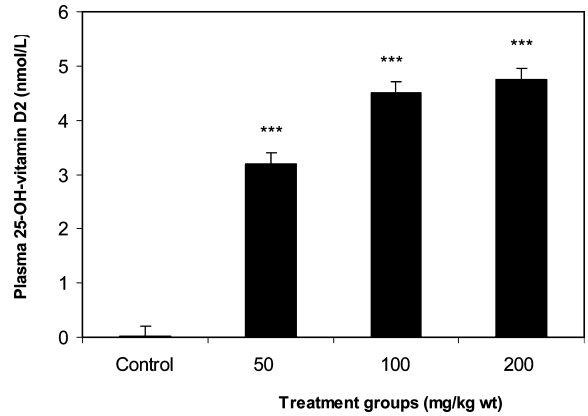

Figure 4. Plasma 25-hydroxyvitamin D2 levels in rats fed UV-C-treated *A. bisporus* mushrooms. Data are means ± SD with five animals per group. Control were rats fed the non-irradiated mushrooms. *** indicates $P < 0.0001$ compared with control group.

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