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Fired Shell Powder of Bivalve *Corbicula Japonica* Improves Mal-Function of Liver—Possible Development of Multi-Functional Calcium

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Abstract: Background: Fired shell powder of bivalve *Corbicula japonica* has been traditionally used in Japan as a folk medicine to improve the liver disorder without any scientific evidences. This experiment was designed to ascertain an anti-hepatitis activity of the fired shell powder of the *Corbicula japonica*. **Methods:** Shell of the *Corbicula japonica* was fired to analyze a crystal structure by the X-ray diffraction apparatus, followed by animal tests to understand relation of the crystal structure of CaCO₃ and their bio-functions. Bio-functional tests were carried out on the anti-hepatitis act by hepatitis model of the LEC (Long Evans Cinnamon) rat, and on the immune activation, lipid controlling, anti-alcoholic damage activity, and liver cells proliferation potency by the C57BL/6 mouse model. **Results:** The fired shell powder of the *Corbicula japonica* calcite, which was prepared by firing at 500°C for 2 hrs, demonstrated the liver function improving activity in the hepatitis model of the orally administered LEC rat. The calcite fed-LEC rats prolonged their survival period by the improving acute hepatitis symptoms, however, the *Corbicula japonica* aragonite and *Ruditapes philippinarum* calcite did not work effectively. Additional activities of the *Corbicula japonica* calcite were lowering GOT <AST> (glutamic oxaloacetic transaminase), GPT <ALT> (glutamic pyruvic transaminase), TBil (total bilirubin), and IAP (immunosuppressive acidic protein) value in serum, enforcement of cellular immune by increasing NK (natural killer) cells activity along with generation of cytokines such as TNF- α (tumor necrosis factor), IL-2 (interleukin), and IFN- γ (interferon) in spleen cells cultivation assay. In the alcohol-fed test, the *Corbicula japonica* calcite prevented alcoholic damages in liver, and also lowered lipid level in serum. **Conclusion:** The *Corbicula japonica* calcite prepared by firing shell demonstrated a wide range of bio-activities as described above, which will surely contribute to normalize liver disorder accompanying with other types of bio-activities.

Key words: Multi-functional calcium, fired shell powder of *Corbicula japonica* calcite, liver function improvement, NK cells activation, cytokines, liver cells proliferation, lowering lipid level.

1. Introduction

Hepatitis is an inflammation of the liver that is most commonly caused by viruses but may also be due to chemicals, drugs, alcohol, inherited diseases, or autoimmune disease. The inflammation can be acute, flaring up and then resolving within a few weeks to months, or chronic, enduring over many years. Chronic hepatitis may persist for 20 years or more before causing significant symptoms related to

progressive liver damage such as cirrhosis, liver cancer, or death. Number of patients with liver disorder is now yearly increasing world widely due to virus mediated and intake of much alcoholic beverage such as a rice wine in Japan to evacuate from the stressful life.

The bivalve *Corbicula japonica* meat (extracts) has been traditionally used for a long period in Japan as a folk medicine to improve the functional disorder in liver without any scientific evidences until recently. Our laboratory had lately attested to the anti-hepatitis efficiency of the *Corbicula japonica* meat extracts [1, 2] by the LEC (Long Evans Cinnamon) rat hepatitis

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model, which develops an acute hepatitis within 6 months old due to the hereditary defect [3]. However, we regrettably have no scientific datum to prove a liver function improvement activity in the fired shell powder of the *Corbicula japonica*, which has been still using likely to be the *Corbicula japonica* meat extracts in Japan. When we could obtain a bio-functional evidence in the fired shell powder (not meat extracts) against the liver disorder and the like, it is much advantageous to use it for maintaining health conditions together with reusing worthless shells as a source of calcium supplement.

Calcium is an essential mineral component to keep a wellness in lifestyle, and ninety-nine percent of it is stored in the bones and teeth. The rest of calcium in the body has other important usages, such as some exocytosis, especially neurotransmitter release, and muscle contraction. Long term calcium deficiency can lead to rickets and poor blood clotting and in case of a menopausal woman, it can lead to osteoporosis, in which the bone deteriorates and there is an increased risk of fractures. The above described activities are our general understanding on the calcium functions, and calcium is at the moment mainly discussing from a nutritional aspect like daily amount of intake to prevent development of the above mentioned diseases. However, an additional novel bio-function(s) of calcium has not been stated up to now. Our concern was, therefore, toward this fascinating subject to cultivate a new bio-function(s) of calcium by using the fired shell powder (CaCO_3) of the *Corbicula japonica*. We introduce the novel bio-functions of calcium such as the anti-hepatitis activity, enforcement of cellular immune system, liver cell proliferation potency and lowering serum lipid in the calcite fed-animal that had not been reported in the past.

2. Materials and Methods

2.1 Firing Conditions of Shells

The fresh shell fish *Corbicula japonica* living in blackish water was boiled in water to remove meats [4]

(Fig. 1). After rinsing the shells in distilled water, they were dried at room temperature for 2 days. The firing conditions of the shells were at from 105 to 900°C for 2 hrs by the programmed temperature control machine (AT-E 58, Isuzu Co. Ltd., Japan). Then, the fired samples were milled to powder by the machine (T-100, Kawasaki Heavy Industry, Japan), thereafter, they were filtrated by mesh with 45 μm pore size. Crystal structure of the fired shell powder (CaCO_3) was analyzed by the X-ray diffraction apparatus (XD-610, Shimazu, Japan). Shell fish of *Ruditapes philippinarum* and *Mizuhopecten yessoensis* was used as a reference.

2.2 LEC Rats

One- to two-month-old female LEC rats (200-250 g) as a hepatitis model were purchased from the Charles-River Co. Ltd., Tokyo Japan for the anti-hepatitis test of the fired shell powder. This animal is characteristic in development of the hereditary hepatitis on around 4 months old, and many of them die of an acute hepatitis at this stage. Five LEC rats formed a group, and the number of groups varied depending on the types of experiments performed. To determine the bio-functions and effective dosage of the *Corbicula japonica* calcite, LEC rat was daily fed powder of the *Corbicula japonica* calcite with the two different dosages of 7 or



Fig. 1 *Corbicula japonica* used for the experiments has a 2-3 cm black shell in diameter, and is living in blackish water.

14 mg/1.0 mL DDW (double distilled water) by catheter until the end of experiment. Blood was collected each month for analyses of GOT <AST>, GPT <ALT>, and TBil value in serum by the enzyme analyzer (FUJI DRI-CHEM, Japan), and IAP (immunosuppressive acidic protein) was determined by the single radial immuno-diffusion method (Saikin-Kagaku Kenkyusho, Sendai, Japan).

2.3 C57BL/6 Mice

Two-month-old female mice (18-22 g) were obtained from the Animal Laboratory Center, Shanghai China to examine the immune stimulating potency of the *Corbicula japonica* calcite. Five mice formed a group, and the number of experimental groups prepared was adjusted depending on the types of experiments designed. In cytokine analyses, each animal was orally given the *Corbicula japonica* calcite at the 1.0 mg/0.1 mL DDW/mouse/day or 5.0 mg/0.5 mL DDW/mouse/day by catheter for serial 12 days, then feeding was ceased for the test of cytokine production. On day first after feeding-stop of the fired shell powder, 5 mice in each group were daily sacrificed to collect test samples such as sera and spleen cells to know how long the effect of the *Corbicula japonica* calcite lasts after feeding-stop. The collected sera were kept at -20°C until use.

These animal tests were conducted on the Approvals of the Animal Ethics Committee of Hirosaki University in Japan and China Medical University in China respectively, stating to avoid an excess distress burden to the animals through the experiments. The control mice were fed distilled water instead of the shell powder under the same experimental manner. Detailed animal design for the tests was described in the individual section.

2.4 NK (Natural Killer) Cells Activity in the *Corbicula Japonica* Calcite-Fed C57BL/6 Mice

The NK cells activity in the cultured spleen cells was tested by the LDH release assay system (lactate

dehydrogenase; Sigma, USA). The spleen cells for cultivation were prepared from the *Corbicula japonica* calcite-fed mice (n=5), which were orally given the 1.0 mg/0.1 mL DDW/mouse/day by catheter for 12 days, and sacrificed to isolate spleen cells. Ratio for the co-cultivation of the spleen cells and Yac-1 cells was at 100:1 in the 10.0% FCS (inactivated fetal calf serum) RPMI-1640 medium (Gibco, USA). OD value of the cultured supernatants was measured at 570 nm after the 48 hrs incubation at 37°C under 5.0% CO₂-air, and the specific Yac-1 cells lysis (NK cells activity) was expressed by the following formula:

Specific lysis = [(experimental release-spontaneous release)/(100% release-spontaneous release)] × 100

2.5 Cytokine Measurement in the Cultured Supernatant of Spleen Cells Prepared from the *Corbicula Japonica* Calcite-Fed C57BL/6 Mice

Mice (n=5) were orally administered of the *Corbicula japonica* calcite with the dosage of the 1.0 mg/0.1 mL DDW/mouse/day by catheter for 12 days. Isolated spleen cells adjusted at 1.0×10⁷ mL in the 10.0% FCS RPMI-1640 medium were incubated at 37°C under 5.0% CO₂-air condition for 48 hrs. Cytokines (TNF-α, IL-2, and IFN-γ) in the cultured supernatants were respectively measured by the ELISA kits (R&D, USA), and expressed as an average value from the triplicate tests.

2.6 Liver Cells Proliferation Test in the *Corbicula Japonica* Calcite-Fed C57BL/6 Mice

The fired shell powder at the 1.0% (1.0 mg/0.1 mL DDW/mouse/day) or 5.0% (5.0 mg/0.5 mL DDW/mouse/day) was orally given by catheter for 12 days. Control animal was treated with DDW alone in the same experimental manner. The separated liver was digested by the 0.1% trypsin in PBS (phosphate buffer solution, pH 7.4), and the number of liver cells was adjusted to 1.0×10⁶ mL in the 10% FCS RPMI-1640 medium. Hundred μL of the liver cells suspension/well was incubated in the 96 multi-well plate at 37°C in

5.0% CO₂ for 48 hrs. Then, the 20.0 uL MTS/PMS (20:1) (MTS;[3-(4,5-dimethylthiazol-2-yl)-5-(3-caboxymethoxyphenyl)-2(4-sulphophenyl)-2H-tetrazolium, inner salt]/PMS; phenazine methosulfate) was added into each well, and incubated additional 4 hrs to measure OD value at 492 nm. The survival rate of the cultured liver cells was measured by the MTS method (Promega KK, Tokyo Japan), and expressed by the following formula:

$$\text{Survival rate (\%)} = \frac{[\text{OD value in tested group (calcite fed)} - \text{OD value in blank}]}{[\text{OD value in control group (non calcite fed)} - \text{OD value in blank}]} \times 100\%$$

2.7 Lipid Lowering in the Serum of *Corbicula Japonica* Calcite-fed C57BL/6 Mice

Mice (n=5) were raised with standard diet pellet with (or without) the fired shell powder at the 5.0 mg/0.5 mL/mouse/day (oral administration by catheter) for 12 days. Serum was isolated to measure the lipid amount by the automatic biochemical analyzer (7060, Hitachi, Japan).

2.8 Alcoholic Damage-Prevention in the Liver of the *Corbicula Japonica* Calcite-Fed C57BL/6 Mice

Ten percent ethanol (1.0 mL/mouse/day) was orally administered by catheter for 12 days with (or without) the 1.0 mg of the *Corbicula japonica* calcite powder. The removed liver was histo-pathologically examined to evaluate the effects of the fired shell powder against an alcoholic damage in the liver.

2.9 Statistical Analysis

The Student's *t*-test (SPSS for Windows 16.0) was employed for the evaluation of the statistical significance.

3. Results

3.1 Crystal Structure of the Fired Shell Powder (CaCO₃)

The crystal structure of the fired shell powder of the *Corbicula japonica*, *Ruditapes philippinarum* or

Mizuhopecten yessoensis was unanimously an aragonite type in structure as fired at below 250°C, a calcite at 500°C, and they evenly became a lime at over the 750°C treatment (Table 1). Major chemical constituent of the *Corbicula japonica* calcite was calcium at 98%, and toxic heavy metals such as arsenic and lead were undetectable (Table 2).

3.2 Anti-hepatitis Activity of the Fired Shell Powder

In the preliminary tests of anti-hepatitis activity of these fired shell powder, the *Corbicula japonica* calcite worked most effectively to improve the liver disorder. Neither the aragonite of the *Corbicula japonica* nor the calcite of the *Mizuhopecten yessoensis* showed any efficacy for improvement of the liver function (Table 3). Based on these results, the *Corbicula japonica* calcite was selected to use for the further detailed experiments.

3.3 Prolonged Survival Time in the *Corbicula Japonica* Calcite-Fed LEC Rats

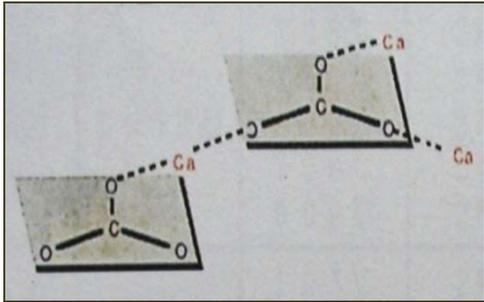
The *Corbicula japonica* calcite-fed rats prolonged their survival periods compared with these of the calcite non-fed control rats. The survival rate in the 14mg-fed rats was at 80% at 7 months old age, whereas, those of both the 7 mg-fed and control group were at 60% respectively at the same period. No adverse events were clinically observed in the 14 mg-fed rats with a stable increase of body weight for 3 months feeding (not shown).

3.4 Improvement of Liver Function in the *Corbicula Japonica* Calcite-Fed LEC Rats

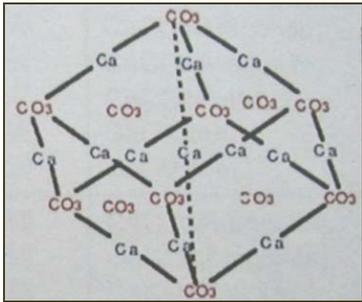
Each of liver function makers GOT, GPT or IAP showed low level in the sera of the *Corbicula japonica* calcite-fed LEC rats (Fig. 2), indicating the presence of the bio-functional efficacy of calcite to restore liver function in disorder. The fourteen milligram of the *Corbicula japonica* calcite was thought to be a recommendable dosage to recover mal-function of liver in the LEC rat hepatitis model.

Table 1 X-ray diffraction analysis of the fired shell powder.

Shell fish	Firing condition	Structure
<i>Corbicula japonica</i>	105°C 2hrs	CaCO ₃ (Aragonite)
	250 2hrs	CaCO ₃ (Aragonite)
	500 2hrs	CaCO ₃ (Calcite)
	750 2hrs	CaO(Lime)
	900 2hrs	CaO(Lime)
<i>Ruditapes philippinarum</i>	105°C 2hrs	CaCO ₃ (Aragonite)
	250 2hrs	CaCO ₃ (Aragonite)
	500 2hrs	CaCO ₃ (Calcite)
	750 2hrs	CaO(Lime)
	900 2hrs	CaO(Lime)
<i>Mizuhopecten yessoensis</i>	105°C 2hrs	CaCO ₃ (Calcite like)
	250 2hrs	CaCO ₃ (Calcite like)
	500 2hrs	CaCO ₃ (Calcite)
	750 2hrs	CaO(Lime)
	900 2hrs	CaO(Lime)



(Aragonite)



(Calcite)

Structural illustration of the aragonite and calcite

3.5 Increasing NK Cells Activity and Cytokines Production in the Cultured Spleen Cells Prepared from the *Corbicula Japonica* Calcite-Fed Mice

The NK cells activity in the cultured spleen cells increased to reach maximum on day 2nd after ceasing the calcite feeding (Fig. 3). Increasing amount of TNF- α was also detected in the culture supernatants of spleen cells, and became maximum ($p < 0.05$ against control value) on day 4th. Other cytokines of IL-2 and IFN- γ were slightly increased, but not statistically significant (Table 4).

3.6 Liver Cells Proliferation in the *Corbicula Japonica* Calcite-Fed C57BL/6 Mice

Liver cells from the *Corbicula japonica* calcite-fed C57BL/6 mice substantially proliferated in the cells

culture analysis (Fig. 4), and it became 300% at a proliferation rate in the 1.0% (1.0 mg) of the *Corbicula japonica* calcite-fed mice group against that of control group value in incubation for 36 hrs. That of the 5.0 % (5.0 mg) calcite-fed group also increased, however, it was less activity compared to that of the 1.0% calcite-fed group.

3.7 Lipid Reduction in the *Corbicula Japonica* Calcite-Fed C57BL/6 Mice

TG (triglyceride) and TC (total cholesterol) value in serum decreased in the *Corbicula japonica* calcite-fed mice. Especially TC level was significantly low ($p < 0.05$), along with lowering both the HDL (high density lipoprotein cholesterol) and LDL (low density lipoprotein cholesterol) level (Table 5).

Fired Shell Powder of Bivalve *Corbicula Japonica* Improves Mal-Function of Liver –Possible Development of Multi-Functional Calcium

Table 2 Chemical constituents of the *Corbicula japonica* calcite.

Element	Amount
Ash	99.8%
Carbohydrate	0
Protein	0
Lipid	0
Ca	45,766 mg (98%)
As	<3 ppm
Pb	<10 ppm
Mg	8 mg
Na	649 mg
K	48 mg
P	101 mg
Fe	5 mg
Zn	3 mg/100 g
Cu	0.11 mg/100 g



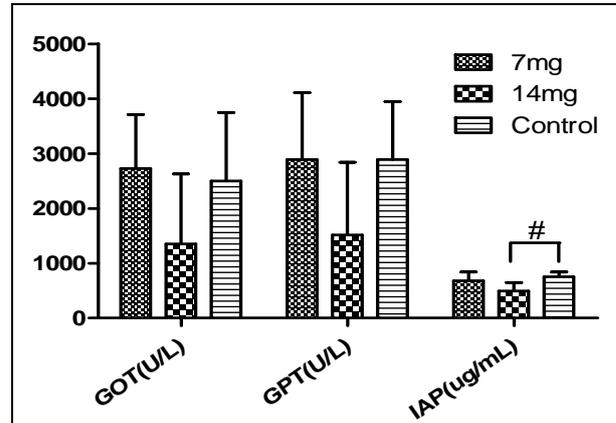
Fired shell powder prepared from the *Corbicula japonica* calcite

Table 3 Preliminary study to examine bio-effects of the aragonite and calcite prepared from two types of shell fishes against the LEC rat hepatitis model.

Sample	TBil(mg/dL)	GOT(U/L)	GPT(U/L)
<i>Corbicula japonica</i> (Aragonite)	1.1±1.0	336±222	309±270
<i>Corbicula japonica</i> (Calcite)	0.5±0.3	232±102	211±180
<i>Patinopecten philipinarum</i> (Calcite)	4.2±5.3	453±246	404±261
Control (Water)	0.8±0.3	268±95	254±136

TBil: Total bilirubin, GOT <AST>: Glutamic oxaloacetic transaminase, GPT <ALT>: Glutamic pyruvic transaminase.

LEC rat was fed the powder of the *Corbicula japonica* calcite at dosages of 14 mg/1.0 mL DDW (double distilled water) by catheter for one month to determine efficacy. Results obtained indicated protective efficacy of the *Corbicula japonica* calcite by lowering liver function markers.



IAP: Immunosuppressive acidic protein

Fig. 2 Improvement of liver function in the *Corbicula japonica* calcite-fed LEC rats. LEC rat was fed the test sample for 3 months to evaluate efficacy of the *Corbicula japonica* calcite. Fourteen milligram per rat was an effective dosage to lower liver function makers. Individual marker indicated low value suggesting anti-hepatitis efficacy of the 14 mg calcite feeding.

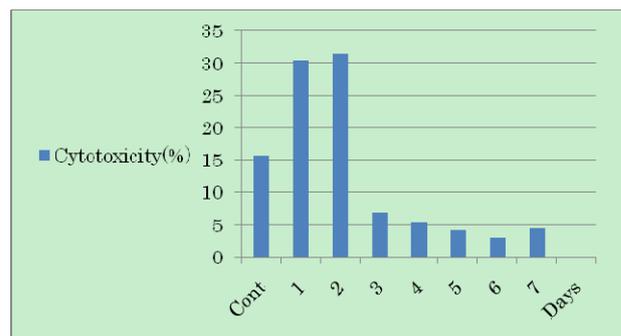


Fig. 3 Increased NK cells activity in the *Corbicula japonica* calcite-fed LEC rat spleen cells. Mouse was orally given the 1.0 mg of *Corbicula japonica* calcite/0.1 mL DDW/mouse/day by catheter for 12 days, and sacrificed for spleen cells cultivation. NK cells activity in spleen cells cultivation increased to reach maximum on day 2nd after ceasing the calcite feeding.

3.8 Efficiency of the *Corbicula Japonica* Calcite against Alcoholic Damage in Liver

In the pathological studies, the *Corbicula japonica* calcite protectively worked against the alcoholic damage, and liver cells kept rigid form without degeneration (Fig. 5).

4. Discussion

It is an established fact that calcium is an essential component playing a key role in keeping bones strong

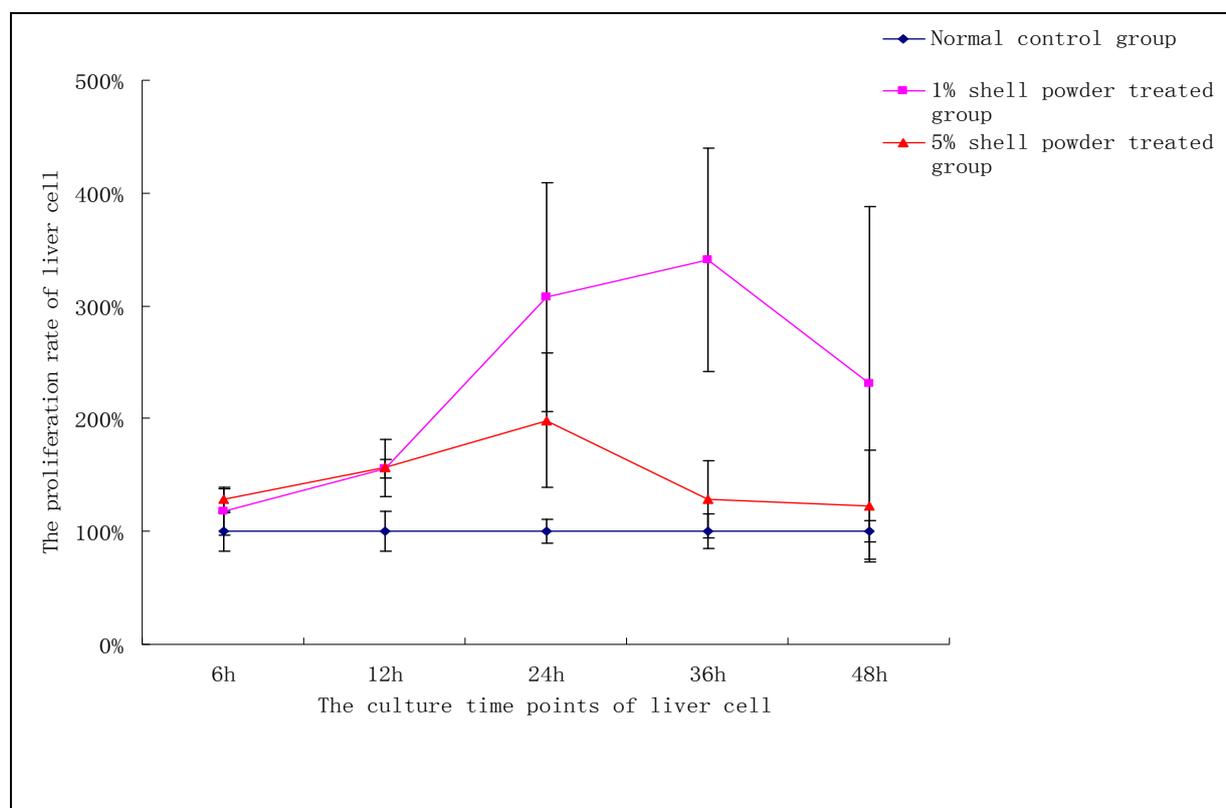


Fig. 4 Liver cells proliferation in the *Corbicula japonica* calcite-fed C57BL/6 mice (n=5).

The fired shell powder at the 1.0% (1.0 mg/0.1 mL DDW/mouse/day) or 5.0% (5.0 mg/0.5 mL DDW/mouse/day) was orally given by catheter for 12 days, and live cells were isolated for analysis of liver cell proliferation. Liver cells from 1% calcite-fed mice (□upper line) well grew, followed by 5% calcite-fed (Δmiddle) to compare with non-calcite fed control liver cells (◇bottom).

Table 4 Cytokine level in culture supernatant of spleen cells isolated from the *Corbicula japonica* calcite-fed C57BL/6 mice.

Days after feed-ceasing	TNF- α (pg/mL)	IL-2 (pg/mL)	IFN- γ (pg/mL)
Control (13 th) (n=5)	27.78 \pm 13.41	15.42 \pm 1.26	12.59 \pm 1.80
Day 1 (13 th) [#] (n=5)	31.86 \pm 10.37	13.33 \pm 1.22	10.72 \pm 1.56
2 (14 th) (n=5)	25.53 \pm 8.79	13.54 \pm 1.82	12.11 \pm 1.01
3 (15 th) (n=5)	29.46 \pm 9.78	13.25 \pm 1.26	11.77 \pm 0.69
4 (16 th) (n=5)	47.29 \pm 6.53*	16.06 \pm 1.14	14.51 \pm 0.60
5 (16 th) (n=5)	25.08 \pm 11.98	12.67 \pm 1.12	13.39 \pm 2.18
6 (17 th) (n=5)	33.31 \pm 9.22	12.88 \pm 1.04	14.63 \pm 1.01
7 (18 th) (n=5)	26.92 \pm 6.96	13.49 \pm 1.13	13.04 \pm 1.44

* p<0.05 (against control), (n): number of mouse used

Calcite (1.0 mg/0.1 ml DDW/mouse/day) was fed for 12 days by catheter, and feeding was ceased from next day (13th) up to the end of experiments (18th). Animals were each day sacrificed for cytokine analyses in supernatants of spleen cells cultivation.

[#]Day 13th indicates; First day after ceasing the *Corbicula japonica* calcite feeding.

and healthy later in life. Other roles well known are controlling blood pressure, muscle contraction, production of enzyme and hormone, and neurotransmitter release. A short of calcium impairs

those functional roles and results in induction of cell death. Immune cells are not exceptional to be affected by calcium, and more sensitive to calcium than other types of cells. Above described functions of calcium

Table 5 Individual lipid amount in sera of the *Corbicula japonica* calcite-fed C57BL/6 mice (m mol/L) (n=5).

Group	TG	TC	HDL-C	LDL-C
Normal diet group	0.86±0.14	2.26±0.43	1.24±0.33	1.29±0.25
Normal diet + shell powder- fed group	0.62±0.20	1.73±0.27*	0.87±0.24	1.05±0.21

p<0.05

TG: Triglyceride, TC: Total cholesterol, HDL-C: High density lipoprotein cholesterol, LDL-C: Low density lipoprotein cholesterol. After feeding the fired shell powder (5.0 mg/0.5 mL/mouse/day) by catheter for 12 days, lipid in serum was measured to evaluate anti-lipid effect of the calcite. All types of lipids decreased in amount in the calcite-fed mice.

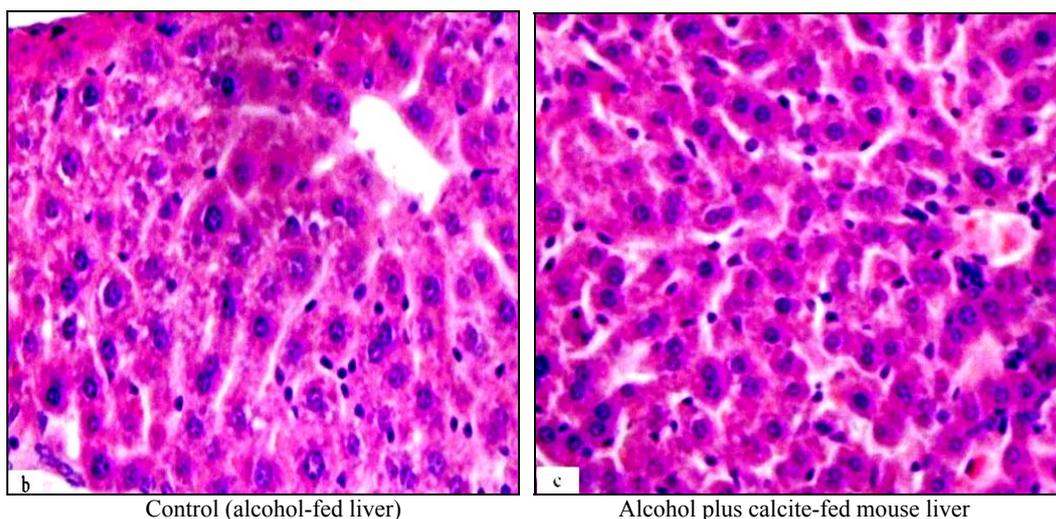


Fig. 5 Protective effect of the *Corbicula japonica* calcite against alcoholic damage in liver. Ten percent ethanol (1.0 mL/mouse/day) was orally administered by catheter for 12 days (with or without) the 1.0 mg of the *Corbicula japonica* calcite powder. Degenerated focus was visible in the alcohol-fed C57BL/6 mouse (left, circled), whereas no focus of alcoholic damages was observed in the alcohol plus *Corbicula japonica* calcite-fed mouse liver (right). (×400)

have already been established in the past, but an additional new bio-action(s) of calcium has not documented until recently.

We here presented the new multi-functional calcium (CaCO₃) which was involving the anti-hepatitis action, NK cells enhancement, cytokines generation, liver cells proliferation and lowering lipid level in serum, which were specifically induced by the *Corbicula japonica* calcite, but not by the *Corbicula japonica* aragonite and not by the *Mizuhopecten yessoensis* calcite. Based on these findings the *Corbicula japonica* calcite was selected for further detailed experiments.

We especially took note of the NK cells activation by the calcite. Because a major role of the NK cells is a survey *in vivo* to find out and eradicate the abnormal cells like the virus infected cells or cancer cells to

maintain a stable healthy state. Actually the foci in the LEC rat hepatitis involve numerous inflammatory cells, occasionally cancer cells and cell's debris, which lead animal to death at a high percentage on the acute hepatitis stage in this model. The activated NK cells efficiently work to eliminate these harmful abnormal cells together with other immune cells like the macrophages. These immune cells are mutually augmented by the cytokines secreted from the activated immune cells. Degenerated liver cells are probably replaced by the new cells to restore normal liver functions by operation of the *Corbicula japonica* calcite (liver cells proliferation), and it eventually results in prolongation of the survival period in the LEC rats (anti-hepatitis activity).

The meta-analysis studies on the interaction between calcium and cancer indicated the moderate

contribution of calcium to the prevention of adenomatous colonic polyps by means that were not yet fully understood [5, 6]. Dietary calcium supplementation also showed preventive work against development of colorectal cancer, adenomatous polyps and calcium metabolisms disorder [7]. Considering together with these reports, we are speculating that the *Corbicula japonica* calcite also has an anti-cancer activity. The cirrhosis, which is generally considered to be the first step of cancer development in liver, was prevented by the calcite feeding in the LEC rat model. Our present concern is now toward how the *Corbicula japonica* calcite works against cancer cells, and to know whether this calcium is applicable as the vehicle of cancer therapy.

There seems to be an interrelation between bio-actions of the *Corbicula japonica* calcite and the crystal structure of calcium, but no acceptable explanation was there at present. Lately, an interesting phenomenon was found in the physical science field that the 500°C heated-iron became much stronger against the impact burden than that of the 1300°C heated-iron due to the change of molecular structure (molecular grating) on the iron surface by heating [8, 9]. It is very suggestive to understand our results, and might be one of persuasive clues to explain why the calcite but not aragonite works effectively against the liver disorder. New bio-functional material(s) will be developed in a mineral or other field of work when this hypothetical thinking is scientifically proved in the future. Our findings reported here were all new phenomena on the calcium's bio-functions that had not been reported previously, therefore, the identifiable experiments should be required to reconfirm our findings using the different criteria especially such as the biophysical techniques that will

contribute to explore a new world in the calcium research field.

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