Effects of Cell-free Supernatants of Yogurts Metabolites on Coxsackie B3 Virus in Vitro and in Vivo

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ABSTRACT

The inhibitory effect and mode of action of cell free supernatants (CFS) of six yogurts metabolites were evaluated against Coxsackie B3 virus using MTT colorimetric and virus-induced cytopathic reduction methods. Results showed that the CFS containing the metabolites of yogurts fermented with probiotic bacteria have high antiviral activities against CVB3 in vitro and in vivo for all the CFS of yogurts metabolites. It may represent a potential therapeutic agent for viral myocarditis and can be used as functional dairy fermented food with a broad therapeutic potential.

Key words: Coxsackie B3 virus, Antiviral activity, Yogurt and Bio-yogurt.

Introduction

Coxsackie viruses are positive strand-RNA viruses that belong to the Picornaviridae family and to the Enterovirus genus. They are classified into groups A and B by their pathogenicity in newborn mice. Group B Coxsackie viruses have been associated with a wide range of human diseases. It can cause fatal viral myocarditis, hepatitis, pancreatitis, meningitis, gastroenteritis, hand, and foot mouth disease. Coxsackie B3 virus is the most common pathogen that causes infection after being taken in orally with contaminated food or water and then multiplies in the intestines and often leading to chronic cardiomyopathy with fibrosis which could progress into heart failure. But the exact mechanism of CVB3-induced damage to myocyte is unknown (Knowlton, 2008).

No effective medications are currently available for treating active myocarditis. Furthermore, interferon and immunoglobulin are able to augment the host-protective immune responses to effectively clear viruses from target tissues. However, the curative effect of these agents has not been well established (Yanyan Chen et al., 2012). The search for antiviral substances with high efficacy, low toxicity and minor side effects must continue. Growing interest in natural products derived antiviral agents remain the best resource for chemically diverse new lead entities that could serve for future development as potent and safe antiviral agents for modern medicine (Nagai et al., 2011). Dairy fermented products known to contribute to maintain our health for a long time and exhibited broad spectrum antimicrobial properties against bacteria, fungi and several viruses including Herpes viruses (Jansen et al., 1991), HIV (Swart and Meijer, 1994), CMV (Berkhout et al., 1997), Hepatitis HCV and HBV (Swart et al., 1998).

Our previous study showed that CFS of fermented milk metabolites were produce high antiviral potential against human Rotavirus during different phases of infection (Barakat et al., 2014). The objective of the present study was to investigate the possible antiviral effects of Cell free supernatants of yogurts metabolites against Coxsackie B3 virus in vitro and in vivo.

Materials and Methods

Cell Culture, Viruses, and Animals

Hep-2 (human laryngeal carcinoma) cells were routinely grown in Dulbecco’s Modification of Eagle’s Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 0.1% antibiotic-antimyocytic solution. The test medium used for the cytoltericity assay and antiviral assays contained 2% of the serum. Coxsackie B3 virus (CVB3) Nancy strain provided by Dr. Mohamed Nasr and Dr. Shubbada bopegamage Lab., Slovak Medical University, Slovakia and propagated in Hep-2 cells in our laboratory at NRC.

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The virus titer was determined by CPE induced by viral infection and expressed as 50% tissue culture infectious doses/ml (TCID_{50}/ml) by Reed-Muench method (Reed and Muench, 1938), which was 10 sup 6 TCID_{50}/0.1ml. Virus stock was stored at -70°C until use. The tissue culture plates were purchased from Falcon (BD Biosciences, Franklin Lakes, NJ, and USA). MTTR(3-4, 5-dimethylthiazol-2-yi)-2, 5-diphenyltetrazolium bromide) was purchased from Sigma-Chemicals (St Louis, MO, USA) and bacterial yogurt starter was provided by Dairy and Food Department, National Research centre, Egypt.

In vivo experiments were carried out with specific-pathogen-free BALB/c mice, 4-5 week old, obtained from Animal House of National Research Centre, Egypt. All animal research was conducted in accordance with the National Research Centre guidelines.

Production and Preparation of Cell-free supernatants of yogurt metabolites

95 ml of full cream bovine, buffalo and camel milk were initially preheated to 41°C and inoculated with 5 gm of bacterial starter followed by incubated overnight for 1 day at 41°C in incubator. The yogurt formed was kept refrigerated at 4°C (Rashid et al., 2007). Cell-free supernatants (CSF) of the yogurts metabolites were obtained when 10 ml of each samples was diluted with 10 ml of medium to adjust to pH7.0 value and filtered using 0.22 µm syringe filter (Millipore Corp., Bedford, MA, USA) to remove bacterial and other organisms interrupted growth of cell line. The CSF of yogurts metabolites were kept frozen at -20°C (Hwa-Jung et al., 2009).

Production and Preparation of Cell-free supernatants of Bio-yogurt metabolites

Honey was mixed thoroughly with distilled water in ration 1:10. Then 10 ml of solution was mixed with Sterile of 85 ml of preheated full cream milk (Bovine, Buffalo and Camel) at 41o C and 5 g of a mixture of bacterial yogurt starter. The mixture was incubated at 41°C for 24 hr. The inoculation was terminated at pH 4.5 by placing the mixture in ice-bath for 60 min. then placed in refrigerator at 4°C (Shori and Baba, 2011). Cell-free supernatants (CSF) of the yogurts metabolites were obtained as described in the previous section.

Detection of Coxsackie B3 virus using RT-PCR

RNA was extracted from HEP-2 Cells with compound treatment and virus infection by QIAGEN Kit, according to the manufacturer’s instructions. A 5 µl from extracted sample was shocked at 99°C for 5 min. A 15 µl (final volume) mixture containing the following constituents was added to the denatured sample; 2 µl of RT buffer, 0.08 µl of deoxnucleoside triphosphate and 0.25 µl of primer 5’-CCCCGGACTGAAAGTATCAAATA-3’ at a concentration of 2.5 mM of each dNTP. cDNA synthesis was carried out at 42°C for 45 min, then at 99°C for 5 min. A 10 µl from RT-PCR product sample was mixed with 40 µl (final volume) mixture containing the following constituents; 5 µl of 10x PCR buffer, 4 mM MgCl2, 4 µl of deoxnucleoside triphosphate and 0.25µl of each primer 5’-CCCCGGACTGAAAGTATCAAATA-3’(180-199) and 5’-GCAGTATGGATTAGCCGCAT-3’(479-460). Amplification was carried out in 40 cycles, the first cycle of denaturation was carried out at 94°C for 5min, annealing at 53°C for 45s, extension at 72°C for 45s, and the final cycle at 72°C for 5min (Hai-Rong xiong et al., 2012).

Assays of cytotoxicity and antiviral activity

To measure cytotoxicity assay HEP-2 cells were seeded onto a 96-well culture plate at a concentration of 2x10 sup 4 cells per well. Next day, medium was removed and washed with phosphate buffered saline (PBS). The 96-well plates were exposed to CFS (three wells per CFS) in maintenance medium for 2 days at 37°C, in parallel with the virus-infected cell cultures. For each CFS, four wells were used as controls. After 2 days of incubation, cytotoxicity was evaluated by evidence of morphological change was recorded using the CPE scoring system, MTTR Assay and cytotoxicity was presented as % of control. The antiviral activities of the CFS against Coxsackie B3 virus was determined by a CPE reduction method. Subsequently, HEP-2 Confluent 24-well plates were infected with 100µl of stock Coxsackie B1 virus for 90 min at 37°C. Virus was removed and 100µl of different dilutions of tested metabolites were added. Four wells were used for each dilution and 1000µl of maintenance medium added per well. Then plates were incubated until complete CPE observed through 3 days. Antiviral activity was determined by the inhibition of CPE compared to control. The results were transformed into percentages of the controls, and the percent protection achieved by the test metabolite in the virus-infected cells was calculated using formula of Reed and Muench and expressed as a % of control (Flint et al., 2000 & Vijayan et al., 2004).

The mode of action of antiviral activity against Coxsackie B3 Virus

Effect of CSF metabolites before virus infection

Confluent monolayer of HEP-2 cells was grown in 24-well plates and inoculated with 100µl of tested metabolites for 90 min at 37°C in 5% CO2 incubator. Four wells were used for each dilution. The medium was aspirated and 100µl of Coxsackie B1 virus was inoculated and incubated for 90 min at 37°C in 5% CO2.
Effects of CFS of metabolites on Coxsackie B virus infection

The CFS of each yogurt and bio-yogurt were tested for antiviral activity against Coxsackie B3 virus. The CFS of camel bio-yogurt, camel yogurt and bovine bio-yogurt exhibited significant strong inhibitory effects against Coxsackie B3 virus of 95.4%, 91.1%, and 90.0% respectively, and those of buffalo yogurt, bovine yogurt and buffalo bio-yogurt exhibited moderate antiviral activity of 89.9%, 89.2% and 88.3% respectively (Table 1). The CFS of each yogurt and bio-yogurt did not exhibit cytotoxicity in HEp-2 cells at tested concentration (data not shown). The tested metabolites were exhibited a significant inhibitory effects against Coxsackie B3 virus in comparison with control 10^6 TCID_{50}/ml.

Mode of action of antiviral activity of CFS of yogurts metabolites against Coxsackie B3 virus

All tested metabolite were exhibited a weak antiviral activity less than 50% during virus adsorption step except CFS of camel bio-yogurt, bovine yogurt and camel yogurt of 61.3%, 61.2% and 57.2% respectively, and during virus multiplication step, all CFS exhibited moderate antiviral activity up to 81.3%, 69.4%, and 67.2% of CFS of bovine yogurt, camel yogurt and buffalo yogurt, respectively. But during viral attachment step, the antiviral activity was increased more than previous two steps; it is sometimes up to 86.0% and 81.7% of camel and bovine yogurt metabolites (Table 1). The tested metabolites were exhibited a significant inhibitory effects against Coxsackie B3 virus in comparison with control 10^6 TCID_{50}/ml.

Effects of CFS of yogurt metabolites on infected mice with Coxsackie B3 virus in vivo:

Viruses titers of heart tissues in the infected mice were significantly decreased by CFS of yogurt metabolites treatment after 7 days of infection indicating that all extracted metabolites of yogurt have potent antiviral activity in comparison with positive control as shown in (Table 2).
Also, morphological appearance of myocardium cell during histological examination used for easy determines the severity of damage or inflammation before and after treatment as shown in next Fig 1. Heart tissues were collected from each group on day 7 after infection. Stained with Hematoxylin and Eosin and investigate histological changes and scoring for myocarditis. These photographs for the histological changes were taken by SPOT software for windows.

**Table 1:** Antiviral activity and mode of action of yogurt metabolites against Coxsackie B<sub>3</sub> virus

<table>
<thead>
<tr>
<th>CFS of Yogurt</th>
<th>% Inhibitory Activity (mean ± S.D) against Coxsackie B&lt;sub&gt;3&lt;/sub&gt; virus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antiviral Activity</td>
</tr>
<tr>
<td>Bovine Yogurt</td>
<td>89.2 ± 1.66</td>
</tr>
<tr>
<td>Buffalo Yogurt</td>
<td>89.9 ± 2.53</td>
</tr>
<tr>
<td>Camel Yogurt</td>
<td>91.1 ± 1.34</td>
</tr>
<tr>
<td>Bovine bio-yogurt</td>
<td>90.0 ± 4.11</td>
</tr>
<tr>
<td>Buffalo bio-yogurt</td>
<td>88.3 ± 2.90</td>
</tr>
<tr>
<td>Camel bio-yogurt</td>
<td>95.4 ± 2.31</td>
</tr>
</tbody>
</table>

Values represent the means of three independent experiments. Antiviral activity was presented as % of control.

**Table 2:** Effects of CFS of yogurt metabolites on Coxsackie B<sub>3</sub> virus titer in Vivo

<table>
<thead>
<tr>
<th>Groups</th>
<th>Viral Titer (log TCID&lt;sub&gt;50&lt;/sub&gt;/0.1ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 (Normal)</td>
<td>0.00</td>
</tr>
<tr>
<td>Group 2 (Treated)</td>
<td>4.20</td>
</tr>
<tr>
<td>Group 3 (Positive)</td>
<td>7.00</td>
</tr>
<tr>
<td>Group 4 Bovine Yogurt</td>
<td>2.33</td>
</tr>
<tr>
<td>Group 5 Buffalo Yogurt</td>
<td>4.59</td>
</tr>
<tr>
<td>Group 6 Camel Yogurt</td>
<td>1.96</td>
</tr>
<tr>
<td>Group 7 Bovine bio-yogurt</td>
<td>2.55</td>
</tr>
<tr>
<td>Group 8 Buffalo bio-yogurt</td>
<td>5.10</td>
</tr>
<tr>
<td>Group 9 Camel bio-yogurt</td>
<td>2.78</td>
</tr>
</tbody>
</table>

*All viral titers data in a format of log TCID<sub>50</sub>/0.1ml and viral control titer before injection 10<sup>6</sup> TCID 50/ml*

**Fig. 1:** Microscope changes of H&E stained heart tissue sections in mice.  
* A: Normal appearance myocardium showed that the structural arrangement was clear, that the cell nucleolus was obvious and clear, and that the cytoplasm was enriched. The cell membrane held integrity and there were no infiltrating cells among cardiac fibrin. Compared with the infected control group, the damage of myocardium was relieved and scores of necrosis and infiltration were decreased significantly with treatment of the metabolites.  
* B: Collection of inflammatory cells and Partial fibrosis  
* C: Mild inflammatory cells and Few scattered inflammatory cells.

**Statistical analysis**  
The data were analyzed by SPSS software and expressed as mean S.D. using Student’s t-test and analysis of variance (ANOVA) for survival analysis in vivo experiments.

**Discussion**  
Coxsackie B<sub>3</sub> virus is an important human pathogen inducing acute and chronic viral myocarditis in children and young people (Mason, 2003). There are no vaccines or therapeutic agents in clinical use (Hunziker et al., 2004). Currently, only bed rest and supportive therapy are the available treatments for patients with myocarditis. The development of antiviral agents that can protect the heart cells from Coxsackie B<sub>3</sub> virus infection is of importance to the future therapeutic treatment of viral myocarditis (Gauntt, and Huber, 2003).

In this study, the antiviral activity of CFS of yogurts metabolites against Coxsackie B<sub>3</sub> virus was demonstrated in both in vitro and in vivo. Our results indicate that the metabolites are able to inhibit viral infection in vitro. CFS of camel bio-yogurt metabolite, camel yogurt metabolite and bovine bio-yogurt exhibited the highest percentage of antiviral activity against Coxsackie B<sub>3</sub> virus given 95.4%, 91.1% and 90%, respectively.
respectively. While moderate antiviral activity was observed for CFS of buffalo yogurt metabolite (89.9%), bovine yogurt metabolite (89.2%) and buffalo bio-yogurt metabolite (88.3%). Each metabolite treatment was suppressed the severity of viral CPE while the delayed CPE development in metabolite-treated cells suggests that the metabolites could have more than one mode of action. These results are contrary to previous study that had similar study on Coxsackie virus and showed that the antiviral activity of yogurt metabolites against Coxsackie B3 and Coxsackie B4 were 88% and 81%, respectively and suggested that yogurt containing metabolites fermented with probiotic bacteria may be possess strong antiviral material against RNA viruses (Hwa-Jung et al., 2009).

In subsequent experiments, all CFS of yogurt metabolites exhibited mild to weak antiviral activity during viral adsorption process such as camel bio-yogurt metabolite (62%), bovine yogurt metabolite (61%), buffalo yogurt metabolite (58%), and camel yogurt metabolite (58%), while other metabolites were exhibited a percentage of antiviral activity less than 50%. These records were demonstrated that the metabolites have mild capability to protect the cells from viral infection with percentage less than 60% except two metabolites, camel bio-yogurt metabolite and bovine yogurt metabolite and during viral multiplication process, CFS of, bovine yogurt metabolite, camel yogurt metabolite and buffalo yogurt metabolite were exhibited moderate antiviral activity with percentages (81.3%), (69.4%), and (67.2%) respectively, while other metabolites exhibited inhibitory effect less than 50%. This demonstrates that the metabolites have moderate ability to treat the viral infection during multiplication processes with percentage up to 81%. Finally, during viral attachment process, high to moderate percentages of inhibitory effects were observed for camel yogurt metabolite (86%), bovine yogurt metabolite (81.7%) buffalo yogurt metabolite (71%) and camel bio-yogurt metabolite (70%), while the others were exhibited moderate antiviral activity less than 70%.

Based on the results of this study, we imply that CFS of yogurt, and bio-yogurt metabolites had significant inhibitory effects on Coxsackie B3 biological synthesis process, directly inactivate virus and blocking adsorption to the cells in vitro. Our results are contrary to previous results on antiviral activity of bovine lactoferrin against Poliovirus, Enterovirus which provided that bovine Lactoferrin was an excellent candidate in the search of natural agents against viral enteric diseases, as it mainly acts by hindering adsorption and internalization into cells through specific binding to cell receptors and/or viral particles (Lucilla Seganti et al., 2004).

In vivo, the CFS of each yogurt metabolites presented favorable antiviral activity after CVB3 infection. Our results confirmed that the oral administration of yogurt and bio-yogurt metabolites can protect against Coxsackie B3 infection in vivo. CFS of yogurt and bio-yogurt metabolites treated mice showed that virus titers and histopathological scores of heart tissues were significantly decreased compared with positive controls. Fibrosis and mononuclear cell infiltration caused by CVB3 infection were also significantly alleviated by CFS of yogurt metabolites treatment, indicating that the CFS of yogurt metabolites has potent antiviral activity against Cox B3. Thus, it may represent a potential therapeutic agent against viral myocarditis.

Conclusion

Fermented milk especially camel and bovine milk can be considered as a good food of high nutritive and therapeutic applications. Yogurt fermented with probiotic bacterial may possess strong antiviral materials against Coxsackie B3 Virus. Further studies are necessary to isolate specific antiviral compounds and further research is needed to investigate the effects of CFS of yogurt metabolites against other viruses in vitro and in vivo for the development of effective antiviral drugs.

References


