ANTICANCER EFFECT OF GOAT MILK FERMENTED BY *LACTOBACILLUS PLANTARUM* AND *LACTOBACILLUS PARACASEI*

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ABSTRACT

Fermented milk proteins have been reported to be used for prevention and treatment of various diseases. This study was designed to investigate the anticancer effect of goat milk fermented by *Lactobacillus plantarum* and *Lactobacillus paracasei. In vitro* anticancer activity was studied using HeLa cell lines. HeLa cells were treated with milk hydrolysates and incubated at 37°C for 4 h and the cell viability assayed by MTT (expressed as percentage over control) decreased with the concentration of goat milk hydrolysate. HeLa cells were treated with different (30-240 µg of protein) concentration of goat milk hydrolysate and viability was assessed after 4h. It was observed that the cell viability decreased with increased concentration of goat milk hydrolysate.

Keywords: Goat milk hydrolysate, *Lactobacillus plantarum*, *Lactobacillus paracasei*, Anticancer activity.

INTRODUCTION

Milk proteins are precursors of many different biologically active peptides. These peptides are inactive within the sequence of the precursor proteins but can be released by enzymatic proteolysis during intestinal digestion or during food processing[1]. The potential health benefits of milk protein-derived peptides have been a subject of growing commercial interest in the context of health-promoting functional foods. So far, antihypertensive, mineral-binding and anticarcinogenic peptides have been studied the most for their physiological effects. A few commercial developments have been launched on the market and this trend is likely to continue along side with increasing knowledge about the functionalities of the peptides[2]. The optimal exploitation of bioactive peptides for human nutrition and health possesses an exciting scientific and technological challenge, while at the same time offering potential for commercially successful applications. Bioactive peptides can be incorporated in the form of ingredients in functional and novel foods, dietary supplements and even pharmaceuticals with the purpose of delivering specific health benefits[3].

Although chemical and physical treatments may have some influence, proteolysis by naturally occurring enzymes in milk, exogenous enzymes, and enzymes from microbial starters such as lactic acid bacteria can potentially generate bioactive peptides from milk protein precursors during dairy processing. The formation of bioactive peptides by lactic acid bacteria in dairy products is currently being debated. There are only a few reports available, and some of the results are somewhat controversial. Since peptidase activity is intracellular in lactic acid bacteria, it has been claimed that lactic acid bacteria probably contribute after cell lysis, which is considered a rare event in fermented milk due to the short fermentation time[4].

There are interesting data on anticarcinogenic effect of fermented foods showing potential role of lactobacilli in reducing or eliminating precarcinogens and carcinogens in the alimentary canal[5,6,7]. The enzymes β-glucuronidase, azoreductase and nitroreductase, which are present in the intestinal canal, are known to convert procarcinogens to carcinogens[8]. Oral administration of *Lactobacillus rhamnosus* GG was shown to lower the faecal concentration of β -glucuronidase in humans[9] implying a decrease in the conversion of procarcinogens to cancinogens. Fermented milk containing *Lactobacillus acidophilus* given together with fried meat patties significantly lowered the excretion of mutagenic substances compared to ordinary fermented milk with *Lactococcus* fed together with fried meat patties[10]. The processes of fermentation of foods are also reported to reduce the mutagenicity of foods by degrading the mutagenic substances during the process.

Some of probiotic strains have been reported to influence hematological cancers also. *Lactobacillus reuteri* enhanced TNF-induced apoptosis in human chronic myeloid leukemia-derived cells by modulation of NF-κB and MAPK signaling and reduced proteins that mediated cell proliferation (cyclin D1 and COX-2) or inhibited apoptosis (Bcl-2, Bcl-xL)[11]. Chiu described bacterial soluble factors *(LcrS)* secreted by *Lactobacillus casei rhamnosus* which induced apoptosis of human monocytic leukemia-cell line (THP-1)[12]. Meta-analysis of the efficacy of probiotic supplementation in the prevention and treatment of radiation-induced diarrhea showed its beneficial effect in experimental animal studies. However the results of human clinical trials were not consistent and should be well preformed as the randomized placebo-controlled studies[13].

Milk fermented with *Lactobacillus acidophilus LA-2* was demonstrated to suppress faecal mutagenicity in the human intestine. Studies on antimutagenic activity of milk fermented with mixed-cultures of various lactic acid bacteria and yeast, showed that the fermented milks produced with mixed cultures of lactic acid bacteria had a wider range of activity against mutagens than those produced with a single strain of lactic acid bacteria[14]. However, a review by McIntosh[15] concludes that there is only limited data to support the hypothesis that probiotic bacteria are effective in cancer prevention. In the present study, the anticancer activity of goat milk hydrolysate fermented by *Lactobacillus plantarum* and *Lactobacillus paracasei* were studied.

MATERIALS AND METHODS

Isolation and identification of lactic acid bacteria

Lactic acid bacteria were isolated from goat milk and commercially available dairy products such as Aavin cheese, Aavin yogurt and Aavin curd. The isolation was performed by the routine microbiological procedure and inoculation on a solid medium. Selective media for lactic acid bacteria used were de Man Rogosa and Sharpe (MRS)[16]. The colonies of *Lactobacillus plantarum* and *Lactobacillus casei* grown on the selective media were selected for further studies. The sequence of the organisms was deposited in the GenBank nucleotide sequences databases under accession number JQ255472, JQ255473 for *Lactobacillus plantarum* and *Lactobacillus paracasei* respectively.

Fermentation of goat milk

At the start of the fermentation, the selected microorganisms were added to the fermentation medium under sterile conditions in a concentration of 6.3 cfu/ml of *Lactobacillus plantarum* and *Lactobacillus paracasei* respectively. Samples at the start of the fermentation were taken for analysis. Then the inoculated

fermentation medium was incubated at 37 °C for 48 h, after which fermented samples were used for further study[17].

Preparation of hydrolysate

For the determining the anticancer activity of fermented goat milk hydrolysate, the whey fraction was obtained as per the methodology of Ganong[18] and De Boever *et al*[19]. The pH of milk was adjusted to 3.4 by addition of 50% lactic acid, and then the milk was centrifuged at 6000 x g for 10 min; 10N NaOH was added to the supernatant to raise the pH to 8.3, and then the supernatant was centrifuged at 6000 x g for 10 min. The final supernatant was used as the whey fraction[20].

In vitro anti cancer activity

The human cervical cancer cell line (HeLa) was obtained from National Centre for Cell Science (NCCS), Pune, and grown in Eagles minimum essential medium containing 10% fetal bovine serum (FBS). All cells were maintained at 37ºC, 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

Cell treatment procedure

The monolayer cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium with 5% FBS to give final density of $1x10^5$ cells/mL. One hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37ºC, 5% CO_2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of milk sample as follows. It was diluted in serum free medium to produce four concentrations of equivalent protein content[21]. One hundred microlitres per well of each concentration was added to plates to obtain final concentrations of 240, 120, 60 and 30 μg equivalent protein content /mL. The final volume in each well was 200 μL and the plates were incubated at 37ºC, 5% CO_2, 95% air and 100% relative humidity for 48 h. The medium containing without milk samples served as control. Triplicate was maintained for all concentrations.

MTT assay

After 48 h of incubation, 15 μL of MTT (5mg/mL) in phosphate buffered saline (PBS) was added to each well and incubated at 37 ºC for 4 h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 μL of DMSO and then measured the absorbance at 570 nm using micro plate reader[22]. The percentage of cell inhibition was determined using the following formula.

Cell Inhibition (%) = 100- Abs (sample)/Abs (control) X100.

Nonlinear regression graph was plotted between percentage cell inhibition and Log_{10} concentration and IC_{50} was determined using Graph Pad Prism software.

RESULTS

HeLa cells were treated with fermented milk hydrolysates and incubated at 37 °C for 4 h and the cell viability assayed by MTT (expressed as percentage over control) decreased with the concentration of ACE inhibitor (Fig.1). In order to show the concentration dependent action, HeLa cells were treated with different (30-240 μg of protein) concentration of goat milk hydrolysate and viability assessed after 4 h. It was observed that the cell viability decreased with increased concentration of goat milk hydrolysate (Fig.2).
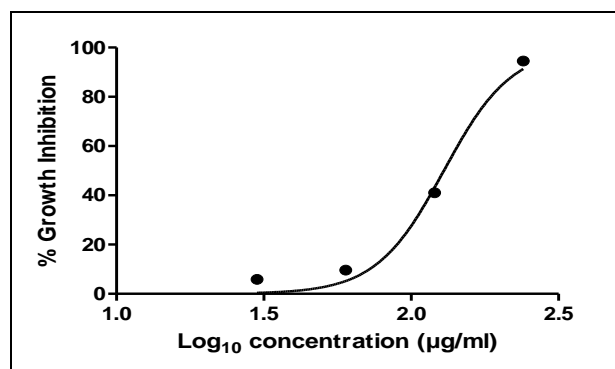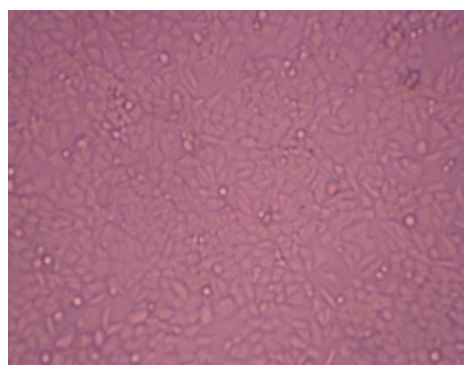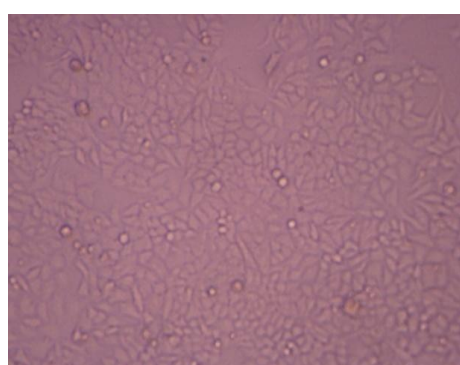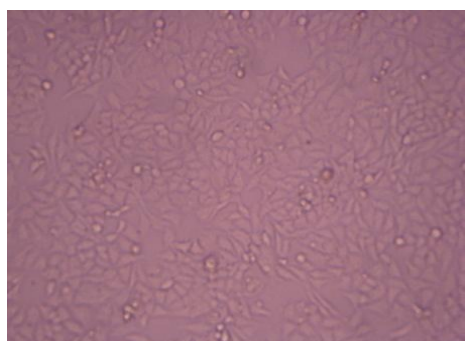

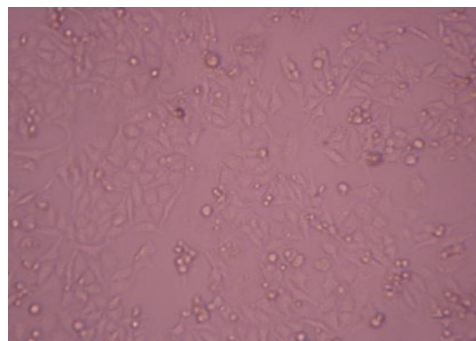
Fig. 1: MTT assay
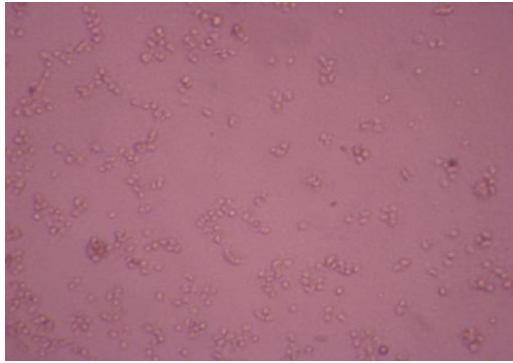

a: Control

b: Cells treated with 30 μg of sample

c: Cells treated with 60 μg of sample

d: Cells treated with 120 μg of sample

e: Cells treated with 240 μg of sample

Fig. 2: *In vitro* anticancer activity of goat milk hydrolysates

DISCUSSION

Williams *et al.*[23] have reported that a proven ACE inhibitor, captopril inhibited the proliferation of HT1080 cell line at a concentration range of 5 to 10 mM which was determined by MTT assay.

Although total intake of dietary protein may be a risk factor for cancer, the type of protein may be the biggest factor in determining any potential anticancer properties of milk[24]. Investigations have been made on the effects of different dietary proteins on tumor incidence in rodents. McIntosh *et al.*[25] demonstrated a protective role for dietary dairy proteins against tumor development, showing that dietary whey protein and casein were more protective against the development of intestinal cancers in rats than was red meat or soy bean protein. They concluded that dietary proteins differ in their ability to protect against cancer development and that the proteins in dairy foods, particularly the whey proteins, appear to play a significant role in cancer prevention. Casein is the major protein in skim milk powder and can display comparative anticancer activity. McIntosh *et al.*[25] found rats that fed on casein had a reduced incidence of DMH-induced colorectal cancer in comparison to rats fed on other dietary sources of protein, although the reduction in dimethyl hydrazine (DMH)-induced colon tumors in comparison to rats fed on red meat or soybean protein was not significant. In comparison, Pence *et al.*[26] found that rats fed on casein as a major dietary protein did have a significantly lower incidence of DMH-induced colon cancer compared to rats fed on cooked lean beef.

In addition to research employing whole whey proteins, some studies have looked at individual whey proteins for their potential anticancer properties. Lactoferrin is an iron binding minor glycoprotein present in bovine milk. A number of physiological roles have been suggested for lactoferrin[27], but it is likely to be the iron-binding properties that contribute to anticancer properties of this whey protein, since free iron may act as a mutagenic promoter by inducing oxidative damage to nucleic acid structure[28]. It is thought that lactoferrin may bind iron locally in tissues, therefore reducing the risk of oxidant-induced carcinogenesis. Sekine *et al.*[29] found that rats fed a basal diet supplemented with 0.2 or 2 % of bovine lactoferrin had incidences of carcinogen-induced colon adenocarcinoma of 25 % and 15 %, respectively, in contrast to 57 % for animals fed a basal (non supplemented) diet. In contrast, iron-saturated lactoferrin was non-protective. Similarly, Tsuda *et al.*[30] looked at the influence of bovine lactoferrin on colon carcinogenesis in rats exposed to carcinogens. The incidences of colon adenocarcinomas in rats receiving lactoferrin were significantly lower than in control groups, and there were also fewer aberrant crypt foci. Smithers *et al.*[31] showed that rats fed a soyabean-based diet supplemented with lactoferrin or with β-lactoglobulin (another whey protein) had significantly fewer aberrant colonic crypt cells (cancer precursors) than animals which consumed the normal (non-supplemented) diet. In addition to its effect in dietary inclusion, there is some evidence that lactoferrin administered by a parenteral route may have important anticancer properties. Yoo *et al.*[32] demonstrated that subcutaneous administration of lactoferrin in its iron-free form could inhibit the metastasis of primary tumors in cancer-bearing mice.

There is now a substantial body of evidence to suggest that bovine milk contains major and minor components which have anticancer properties[33]. The majority of reports which have characterised milk-derived anticancer activity have come from *in vitro* studies using tumor cell lines, or *in vivo* studies using animal models of tumorigenesis. Although both approaches provide valuable evidence as to the potential anticancer actions of milk-derived molecules, caution should be taken when extrapolating results from such studies to statements on disease protection in humans. In the case of *in vitro* studies, the demonstration of an anticancer effect should be taken to imply that the component under test has the potential to regress tumor development (not initiation), and moreover any given biological effect of a component *in vitro* must be assessed in light of its perceived *in vivo* performance in the gastrointestinal tract. This is particularly important with respect to human intestinal physiology: many potentially beneficial molecules in milk may be rendered inactive and remain unabsorbed in the human digestive tract, following gastric processing. The same cautions apply to *in vivo* studies of tumorigenesis in animal models, where the rodent gastrointestinal system may well respond to anticancer factors in a different way to the human digestive system.

Improvements in milk fractionation technology should facilitate the identification of further minor constituents of milk, particularly low-molecular-weight proteins and peptides, which may have important anticancer properties. Such technology has been successfully employed in identifying potent immunomodulatory molecules from milk[34] and there is no reason to suppose that the same technology cannot be applied to the identification and purification of small molecular weight molecules for the appraisal of their anticancer properties.

CONCLUSION

The present study investigated the anticancer properties of fermented goat milk hydrolysates. These findings have suggested that goat milk hydrolysates fermented by *Lactobacillus plantarum* and *Lactobacillus paracasei* may be considered as more promising food component in terms of preventing cancer. However, further study is needed to use this as a therapeutic agent.

ACKNOWLEDGEMENT

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