Research Article

Effects of High Hydrostatic Pressure Processing on the Number of Bacteria and Texture of Beef Liver

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Providing beef liver for raw consumption was banned in Japan on July 1, 2012. To lift the ban, the establishment of effective countermeasures for safe raw consumption is necessary. In this study, we examined the effects of high hydrostatic pressure processing on raw beef liver. Beef liver samples subjected to 300 MPa of pressure or higher for 10 min at 25°C became firmer and showed a paler color and were considered unsuitable for raw consumption. More than 3.0 log reductions of bacteria were seen after treatments at 400 and 500 MPa, but the treatment with lower pressure did not show enough microcidal effects for safe consumption. Histological and ultrastructural analysis revealed that high hydrostatic pressure processing increased mitochondrial swelling and reduced rough endoplasmic reticula in hepatocytes, and such changes might be related to the observed changes of texture in the treated raw beef liver.

1. Introduction

Raw meat dishes, such as “steak tartare,” “mett,” and “yukhoe,” are consumed in many countries, in Europe and Asia. In Japan, raw beef meat and liver, called “Gyu-sashi” and “Reba-sashi,” have/had sometimes been consumed. However, triggered by an outbreak of enterohemorrhagic *Escherichia coli* that occurred on April and May 2011, the Ministry of Health, Labour and Welfare of Japan set a new standard for preparing raw beef on October, 2011. The Ministry imposed a ban on serving raw beef liver at restaurants and meat shops in July, 2012, which will remain in effect until effective countermeasures for safe raw consumption are established [1]. Nevertheless, some people in Japan want the ban lifted to have access to raw beef liver dishes.

Animal offal can be highly contaminated with pathogens, such as enterohemorrhagic *E. coli*, *Campylobacter* spp., and *Salmonella* spp. [2–6]. In fact, many cases of food poisoning have been associated with meat and/or offal

consumption [7–10]. For safe consumption, meat and offal are usually cooked with heat. Heat cooking is quite effective in killing pathogens, but it changes the taste, aroma, and texture of foods. Therefore, alternative processing methods are needed to kill pathogens with minimum changes to taste, aroma, and texture, and thereby allow safe consumption of raw meat and offal.

Radiation [11, 12], high-voltage pulse [13], light pulse [14], high pressure [15], ozone [16], and other methods are known to be effective nonheat sterilization procedures. In particular, high hydrostatic pressure (HHP) processing may be potentially an effective method for reducing the risk of raw beef liver without changing taste, aroma, or texture [17, 18]. So far, a single report describing the effects of high pressure processing on the number of pathogenic bacteria in livestock offal has been found in the literature [19].

In this study, we aimed to examine the effects of HHP processing on bacterial reduction and histological changes in raw beef liver.

2. Materials and Methods

2.1. Preparation of Bacteria. A nonpathogenic *E. coli* strain, ATCC25922, was used instead of enterohemorrhagic *E. coli* O157:H7. The strain was kept at -80°C until use and passaged twice in Trypticase Soy broth (Becton Dickinson and Company, Franklin Lakes, NJ, USA) at 37°C before experiments. The culture was centrifuged at 8,000 rpm for 10 min. Cell pellets were washed and resuspended in sterilized phosphate buffered saline (PBS, pH 7.0). Bacterial cell density was adjusted to 1×10^8 or 1×10^9 colony forming units (CFU)/mL.

2.2. Preparation of Beef Liver Samples. Beef liver, which was taken on the previous day and stored under refrigeration, was purchased from Tokyo Shibaura Zouki Co., Ltd. (Tokyo, Japan), a meat broker company. The beef liver was transferred under cold conditions (using refrigerants) to the laboratory and used on the same day. The beef liver was cut into pieces of approximately $2\text{ cm} \times 3\text{ cm} \times 0.5\text{ cm}$, weighing approximately 10 g. The samples were vacuum-packed in plastic bags twice and sealed using a heat sealer. To evaluate the microcidal effects of HHP treatment, 10 μL of *E. coli* suspension (in PBS), 1×10^8 CFU/mL, was injected in the sample at each of 10 regularly spaced points using micropipette with sterilized pipette tips before being packed.

2.3. HHP Treatment. Beef liver samples in plastic bags were pressurized with a water-based prototype pressurization apparatus (HPV-80C20-S; Sugino Machine Ltd., Toyama, Japan). Pressurization reached 200 MPa after approximately 60 sec, and decompression took approximately 10 sec. The initial temperature before HHP treatment was always at 25°C ; however, the temperature during HHP treatment was not monitored.

2.4. Bacteria Counting after HHP Treatment. The number of *E. coli* in PBS after HHP treatment was determined by the pour culture method in Trypticase Soy agar (TSA, Becton Dickinson and Company). The number of *E. coli* in beef liver after HHP treatment was determined using TSA and chromogenic XM-G agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), a selective agar for coliforms and *E. coli*. After incubation at 37°C for 24 hr, typical colonies with blue color on XM-G agar were identified as presumptive *E. coli*. The number of bacteria in each sample was calculated from the number of colonies multiplied by the dilution factor.

2.5. Experimental Designs

2.5.1. Effects of HHP Treatment on *E. coli* in PBS. Five milliliters of polypropylene tubes (Greiner Bio-One, Kremsmünster, Austria) was filled with *E. coli* suspension in PBS (1×10^9 CFU/mL) and subjected to HHP at 200, 300, 400, and 500 MPa. The treatment was performed for 10, 20, and 30 min at each pressure at 25°C . The bacterial suspension after HHP treatment was serially diluted with sterilized saline supplemented with 0.1% peptone and inoculated on

FIGURE 1: Effects of HHP treatment on *E. coli* in PBS.

TSA plates. The limit of detection for this experiment was 1 CFU/mL. The experiments were repeated 3 times.

2.5.2. Effects of HHP Treatment on *E. coli* Inoculated in Liver Samples. Liver samples inoculated with *E. coli* in plastic bags were submitted to HHP at 200, 300, 400, and 500 MPa for 10 min at 25°C . For *E. coli* colony counting, 10 g of liver sample was homogenized in 90 mL of PBS for 2 min using a Stomacher® 400T circulator (Seward Ltd., West Sussex, UK), serially diluted in PBS, and inoculated on TSA and XM-G agar plates. The limit of detection for this experiment was 10 CFU/g. The experiments were repeated 3 times.

2.5.3. Effects of HHP Treatment on Color, Histology, and Ultrastructure of Liver Samples. Liver samples treated at 200, 300, 400, and 500 MPa for 10 min at 25°C were observed macroscopically and then cut into slices. The colors of the samples were measured by colorimeter (CR-200, Minolta Co., Ltd., Osaka, Japan). Results are expressed as a combination of the values of L^* , a^* , and b^* : L^* represents a gradation from light to dark; a^* , red to green; and b^* , yellow to blue.

For light microscopy analysis, samples were fixed in 10% neutral buffered formalin and embedded in paraffin. Thin sections ($4\mu\text{m}$) were stained with hematoxylin and eosin (HE).

For electron microscopy analysis, small pieces of liver samples were prefixed in MacDowell's and Trump's 4F:1G fixative [20] and postfixed in 1% osmium tetroxide, followed by 0.2 M phosphate buffer, and then embedded in epoxy resin. Using an electron microscope (JEM1011; JEOL Ltd., Tokyo, Japan), ultrathin sections were examined after staining with uranyl acetate and lead citrate.

3. Results

3.1. Effects of HHP Treatment on *E. coli* in PBS. The number of *E. coli* in PBS before HHP treatment was 9.0 log CFU/mL (Figure 1). No significant change was found in the number

TABLE 1: Colorimetric values of liver samples before and after HHP treatment.

Treatment	L^*	a^*	b^*
0.1 MPa	36.7 ± 1.3	6.5 ± 0.6	2.2 ± 0.3
200 MPa	38.1 ± 1.4	6.7 ± 0.5	0.5 ± 0.4
300 MPa	$44.3 \pm 1.1^{\dagger\dagger}$	$10.1 \pm 1.0^{\dagger\dagger}$	2.2 ± 1.4
400 MPa	$47.7 \pm 1.9^{\dagger\dagger}$	$10.9 \pm 0.4^{\dagger\dagger}$	$6.2 \pm 0.7^{\dagger\dagger}$
500 MPa	$50.4 \pm 0.4^{\dagger\dagger}$	$10.1 \pm 0.2^{\dagger\dagger}$	$8.0 \pm 0.6^{\dagger\dagger}$

$^{\dagger\dagger}p < 0.01$.

FIGURE 2: (a) The number of *E. coli* in the liver samples treated with high pressure measured (TSA plates). ** $p < 0.01$ compared with nontreated samples (expressed as 0.1 MPa, because of the atmospheric pressure). (b) The number of *E. coli* in the liver samples treated with high pressure measured (XM-G agar plates). ** $p < 0.01$ compared with nontreated samples (expressed as 0.1 MPa, because of the atmospheric pressure).

of *E. coli* after HHP treatment at 200 MPa for 10 min, but a slight decrease was observed after 20 min and a decrease of 1.0 log in cell number was seen after 30 min HHP treatment. Treatments at higher pressures and longer periods led to more rapid and significant decreases in the number of *E. coli*. Treatment at 500 MPa for 10 min led to a decrease in the number of *E. coli* to 2.5 log CFU/mL, and after 20 or 30 min the number of viable cells was below the detection limit (1 CFU/mL).

3.2. Effects of HHP Treatment on *E. coli* Inoculated in Liver Samples. Figures 2(a) and 2(b) show the number of *E. coli* in the liver samples treated with high pressure counted on TSA and XM-G agar plates, respectively. Nontreated liver samples were expressed as 0.1 MPa in the figures, because of the atmospheric pressure. The number of bacteria spontaneously contaminated in the liver was $5.4 \pm 0.7 \times 10^3$ CFU/g as viable aerobic bacteria on TSA agar plates, $7.0 \pm 3.0 \times 10^1$ CFU/g

as coliform (red colony), and $6.3 \pm 4.5 \times 10^1$ CFU/g as *E. coli* (blue colony) on XM-G agar plates, respectively. The number of *E. coli* in nontreated (*E. coli* inoculated) liver sample was approximately 7.0 log CFU/g. After treatment at 200 MPa, no significant change was observed in the number of *E. coli*. In contrast, a reduction of 1.5, 3.0, and 5.0 log CFU/g was found at 300, 400, and 500 MPa, respectively.

3.3. Effects of HHP Treatment on Color, Histology, and Ultrastructure of Liver Samples. Liver samples in plastic bags after HHP treatment are shown in Figure 3(a); cut surfaces are shown in Figure 3(b). Liver sample volumes did not change after high pressure treatment compared with nontreated samples. The color of liver samples became paler, from reddish brown to whitish brown, under high pressure treatment. Colorimetric results of liver samples are shown in Table 1. The color of liver samples treated with high pressure showed higher L^* , a^* , and b^* values. In addition,

FIGURE 3: (a) Liver samples after HHP treatment. (b) Cut surfaces of liver after HHP treatment.

liver samples submitted to high pressure showed a firmer consistency when cutting. Nontreated liver samples were expressed as 0.1 MPa in the figures and the table, because of the atmospheric pressure.

Histological analysis showed no obvious changes in lobular structures and funicular arrangements of liver cells treated with high pressure (Figure 4(a)) when compared to nontreated samples. However, hepatocytes showed a faintly eosinophilic cytoplasm containing diffusely eosinophilic granules that increased in a pressure-dependent manner (Figure 4(b)). Small eosinophilic particles were also observed in blood vessels (Figure 4(b)).

Ultrastructural analysis showed swollen mitochondria containing dense amorphous granules in hepatocytes (Figure 5(a)). The size of the amorphous granules increased with high pressure treatment (Figure 5(b)), and the rough endoplasmic reticula disappeared completely (Figure 5(c)).

4. Discussion

The Ministry of Health, Labour and Welfare of Japan has banned providing beef liver for raw consumption and requires it to be cooked at 63°C for at least 30 min, or any other heat condition (e.g., at 75°C for at least 1 min) with proven equivalent microcidal effects [21]. However, some people in Japan have been waiting for the establishment of effective countermeasures and for the ministry to lift the ban.

In European countries such as France and Germany, raw ground meat is consumed and is considered a public health concern. Black et al. [22], Hsu et al. [23], and Jiang et al. [24] reported that high pressure processing of ground meat is potentially effective in reducing the risk of ingesting pathogenic bacteria.

Uenaka et al. [19] reported that *E. coli*, *Salmonella* spp., and *Staphylococcus aureus* inoculated into beef liver are killed effectively by high pressure processing (400 MPa for 10 min

FIGURE 4: (a) Histological analysis of liver after HHP treatment (low magnification). (b) Histological analysis of liver after HHP treatment (high magnification).

6 times, or 600 MPa for 30 min once, at room temperature). They also reported that beef liver treated 6 times with 400 MPa for 10 min at room temperature showed a paler color but its taste was similar to that of untreated beef liver as evaluated by sensory tests. In this study, liver samples treated at 300 MPa or higher for 10 min also showed a paler color, and samples treated with 400 MPa or 500 MPa showed a firmer consistency and were not considered raw. No sensory tests were performed in this study. In future studies, the tenderness of treated liver samples will be measured using a rheometer.

Histological and ultrastructural changes of the raw beef liver by HHP treatment were first reported in this study. The changes were more severe in liver samples treated with higher pressure, both histologically and ultrastructurally. The eosinophilic granules seen dispersed in hepatocytes under light microscopy are thought to correspond to the dense amorphous granules observed in mitochondria under electron microscopy. Such changes are thought to underlie

changes in texture of beef liver. The small eosinophilic particles observed in blood vessels are thought to be the debris of red blood cells. The destruction of red blood cells and less eosinophilic cytoplasm of hepatocytes may be related to the paler color of liver treated with high pressure.

We conclude that HHP treatment at 300 MPa or higher for 10 min is unsuitable for raw consumption of beef liver. However, lower pressure treatment did not show enough microcidal effects for safe consumption. In future studies, combinations of shorter/longer duration, single/multiple treatment(s), and lower/higher temperature under lower pressure will be performed to find the optimal conditions to minimize health risks and keep texture of raw beef liver.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

FIGURE 5: (a) Ultrastructural analysis of liver after HHP treatment (hepatocyte). (b) Ultrastructural analysis of liver after HHP treatment (mitochondria). (c) Ultrastructural analysis of liver after HHP treatment (rough endoplasmic reticulum).

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