

# Disinfection of Human Tissues in Orthopedic Surgical Oncology by High Hydrostatic Pressure

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## 1. Introduction

Liquids show significant temperature-dependent compressibility under high hydrostatic pressure (HHP). For instance, the specific volume of water at atmospheric pressure decreases by 12% when exposed to 400 MPa (1). Self-ionization of water is also promoted by HHP lowering the pH phase transition of water is triggered under excessive HHP; at 1,000 MPa water freezes at room temperature, whereas at 207.5 MPa the freezing point can be lowered to  $-22^{\circ}\text{C}$  (1). This allows for pressure shift freezing of foods with instant and small ice crystal formation, storage of food at subzero temperatures without ice formation, or fast thawing of frozen food by pressurization, allowing gentle processing of foods or food constituents with minimal structural damage.

HHP may also cause alterations in biological molecules, associated with a change in their conformation towards of forms which occupy smaller volumes. With increasing pressure, the non-covalent bonds of macromolecules such as proteins are affected leading to changes in their quaternary, tertiary or secondary structure. HHP is presumed to influence the conformational state of lipids as well (2,3) whereas nucleic acids have proved pressure-resistant because their secondary structure is mainly stabilized by H-bonds that are almost pressure insensitive (4;5). HHP-induced changes can be reversible, metastable or irreversible, partly depending on the pressure level itself, but also depending on the duration of the pressure treatment, on the temperature during treatment, on the chemical conditions and on other conditions of the surroundings.

The growth of eukaryotic and prokaryotic cells can be prevented to a large degree by a number of preservation techniques, most of which act by killing the cells or by slowing down cellular growth. Concerning food products, heating, freezing, drying, vacuum packing, acidifying or the addition of preservatives are the predominant method. At present, however, major trends have emerged towards the use of procedures such as HHP to deliver food products that are less 'heavily' preserved but still with high assurance of no microbiological contamination (6,7). HHP as a means of preserving food, without the

addition of any kind of preservative, has attracted increasing attention (4,6,8), since it has the advantage of leaving covalent molecular bonds intact without impairing flavors, aromas, vitamins and other pharmacologically active molecules (9).

In the medical field, HHP technology is now in preclinical testing with the aim of inactivating both pathological microorganisms and tumor cells in resected tissue segments, such as bone, cartilage and tendon *ex vivo* (10-15). This is a promising clinically relevant approach, especially with respect to rapid killing of tumor cells in bone and the subsequent possibility of re-implantation of the once tumor-bearing bone segment back into the patient.

## 2. HHP and orthopaedic surgery

In orthopedic surgery, restoration of bone defects caused by malignant solid tumors is achieved by several methods of treatment such as extracorporeal irradiation or autoclaving the affected bone segment, as an alternative approach to synthetic limb reconstruction (16-19). In contrast, irradiation or autoclaving of osteochondral segments or tendons may lead to severe alteration of their biomechanical and biological properties, a major concern regarding this type of approach (20-22).

A new technology, the administration of short-term HHP to the resected bone segment immediately after surgery, now offers an alternative to the conventional ways of treating tumor-affected bone. At the high pressure value of 600 MPa applied, the biomechanical properties of bones, tendons and cartilage remain unchanged (10-14). Under these conditions, normal eukaryotic cells, and also malignant cells are irreversibly damaged and outgrowth of cells from tumor-afflicted bone and cartilage segments is efficiently blocked (14,15,23).

With regard to the biological properties of treated bone, cartilage or tendon, no obvious changes in the adhesive or growth promoting properties of the extracellular matrix proteins after HHP treatment of the bone were observed (11), and successful revitalization of HHP-treated bone segments *in vitro* was observed. Also, no enhanced activity of proteases, which might be released after HHP treatment of resected human bone tumor and provoke autolytic bone resorption, could be detected (24). This report reviews the basics and technical potential of HHP in orthopaedic surgery and sheds light on the prospects of HHP for the treatment of neoplastic bone and infected bone tissue, cartilage and tendon.

## 3. HHP-device and treatment of affected bone, cartilage, or tendon

The HHP system (Record Maschinenbau, Koenigsee, Germany) consists of a high pressure autoclave, a pressure generation unit, a temperature and pressure control unit and a material handling unit (Figure 1a). HHP treatment of the tissue samples is accomplished by a pressure-transferring medium, usually water, thus allowing uniform and instantaneous transmission of pressure to the biological sample. To treat infected or tumor-afflicted bone, tendon or cartilage, larger specimens are placed into polyethylene bags and sealed by vacuum-packaging (Komet, Plochingen, Germany) (Figure 2). Sealing in buffer is required to assure uniform and instantaneous pressure transmission throughout the biological sample and to prevent contamination while in contact with the

pressure medium. In case of smaller specimens, instead of plastic bags, tissue specimens are placed into 15 ml flexible Falcon tubes (Becton-Dickinson, Heidelberg, Germany) (Figure 3a) or 2 ml Nalgene cryogenic vials (Thermo Fisher Scientific, Wiesbaden, Germany) (Figure 3b). The vials filled with Ringer buffer are carefully capped avoiding air bubbles and then sealed tightly with parafilm (American National Can GmbH, Gelsenkirchen, Germany).



Fig. 1a. High hydrostatic pressure device (Record Maschinenbau, Koenigsee, Germany).

The bags/vials are placed into the central cavity of a water-filled pressure chamber (100 ml) of a custom-made HHP device (Figure 1b). The water is mixed 1:1 with ethylene-glycol to suppress corrosion of the pressure chamber. The temperature of the pressure chamber can



Fig. 1b. The core of the autoclave chamber is made of an amagnetic stainless steel into which a large cylindrical hole has been bored in order to receive the specimen. A metal-on-metal sealing system provides an excellent leak-free closure with minimal mechanical wear. The pressure is transmitted by means of a hydraulic ram and monitored by a pressure gauge. Incubation temperature is monitored by a thermocouple and thermostatic control is ensured by the circulation of water through rubber tubing. On the control panel pressure settings can be adjusted up to 600 MPa).

be adjusted from 0 - 50 °C by a temperature control unit (Thermo Fisher Scientific, Karlsruhe, Germany) (Figure 4). The temperature should be kept constant at any given level since adiabatic compression of water increases the temperature 3 °C per 100 MPa. Pressure levels up to 600 MPa are adjusted manually with a compression / decompression rate of 100-300 MPa/min. The tissue specimens are held under pressure for a defined length of time (plateau phase), then, within a few seconds, pressure is returned to normal.



Fig. 2. Vacuum sealing device (Komet, Plochingen, Germany).

Specimens in polyethylene bags are placed into the vacuum chamber, positioning the open section onto the sealing bar. Once a vacuum is formed a heated wire on the bar seals the plastic bag. On the control board vacuum and sealing settings can be adjusted individually.

Exposure of tendons and ligaments to HHP (300 and 600 MPa; 10 min, 20 °C) did not significantly change their biomechanical features, their Young's modulus or tensile strength, indicating retention of functional properties after HHP-sterilization (12). Retention of biomechanical properties of tissues after HHP is mainly based on the fact that HHP does not affect covalent molecular bonds, leaving parts of the molecule unchanged whereas exposure to chemicals or high temperature often unfold macromolecules irreversibly (25-27).

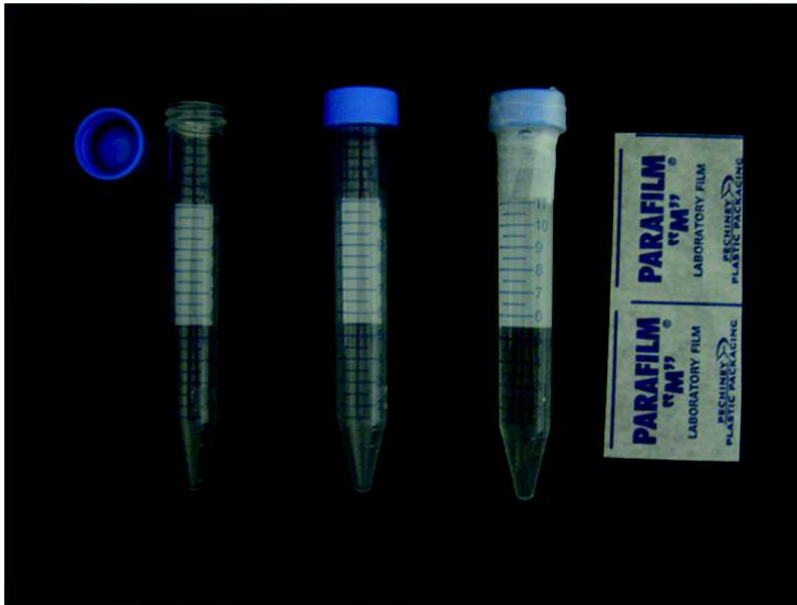


Fig. 3a. Tissue specimens are placed into flexible 15 ml BD Falcon™ conical tubes (Becton-Dickinson, Heidelberg, Germany) or Figure 2



Fig. 3b. 2 ml Nalgene cryogenic vials (Thermo Fisher Scientific, Wiesbaden, Germany) (3b), filled with Ringer buffer and tightly sealed (e. g. with parafilm, American National Can GmbH, Gelsenkirchen, Germany).

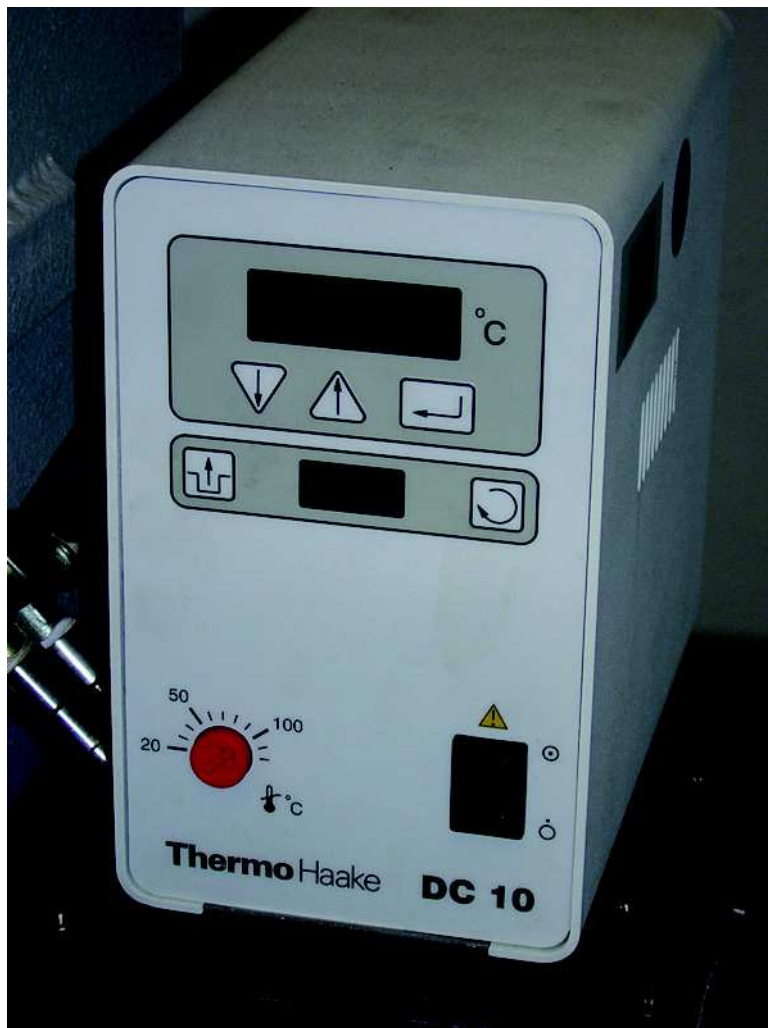


Fig. 4. Temperature control unit (Thermo Fisher Scientific, Karlsruhe, Germany). Open bath circulator with digital settings, bath vessel and bath bridge made of stainless steel and temperature resistant polymer; electronic control using a digital sensor for predetermined reference value, max. temperature 100 °C.

As well as tendons, we have also investigated the biomechanical properties of freshly resected human cortical and trabecular bone specimens or cartilage and menisci exposed to HHP as high as 600 MPa (10 min, 20 °C) (13,28). Under these conditions, no significant alterations relating to the stiffness and relaxation behavior of the osteochondral segments were observed. Unfortunately, inactivation of clinically important bacteria, for instance those present in osteomyelitis was not achieved under these conditions (29,30) although in foods vegetative bacteria, yeasts, and moulds are generally sensitive to pressures of 600 MPa (7).



pre-clinical application of HHP in studies	clinical HHP application	no application in
disinfection of tumor afflicted bone, cartilage, tendons	inactivation of different virus (e.g. HIV) in blood samples	disinfection of inserts of Prosthesis
	food preservation	disinfection of infected tissue

Table 1. The table presents different fields of application of Hygh Hydrostatic Pressure. Save inactivation of bacteria in all kind of materials is not possible at the moment.

HHP has also been employed to investigate pressure-related *in vivo*-effects on chondrocytes since hydrostatic pressure is a significant component of the mechanical loading environment within articular cartilage. Chondrocytes within cartilage of diarthrotic joints experience hydrostatic pressure levels of 0.1-20 MPa (31). In *ex vivo* investigations therefore intermittent high pressure of 10 MPa was applied to investigate mechanisms mediating the response of chondrocytes to joint motion and loading (30,31). Under these conditions, a decreased release of matrix metalloproteases (MMP)-2, tissue inhibitor of matrix metalloproteinase (TIMP)-1 and interleukin-6 by osteoarthritic chondrocytes was observed, suggesting that pressure influences cartilage stability *in vivo* (32).

We have observed that, regarding bone, exposure of normal cells (e.g. osteoblasts) and tumor cells (e.g. osteo-, chondro- and fibrosarcoma cells) to elevated hydrostatic pressure led to irreversibly damaged, non-viable cells, even after short-term exposure to 350 MPa (14,15,23). Under these conditions, eukaryotic cells experience irreversible destruction and permeabilization of cell membranes by HHP causing cell death (33).

Interestingly, suspended tumor cells were more resistant to HHP than adherent tumor cells, yet, normal bone and tissue cells such as fibroblasts and osteoblasts were less resistant to HHP than tumor cells (14,23). We also observed that at 300 MPa *ex vivo* outgrowth of normal or tumor cells from bone ceased concomitant with impairment of the bone-associated cells (15). This finding points to rapid killing of bone-associated tumor cells, potentially allowing re-implantation of the once tumor-bearing bone segment back into the patient.

Looking at other types of cells, Dibb *et al.* investigated the effects of HHP on normal and neoplastic rat cells in culture in the range 0.1 to 150 MPa (34). Morphological changes characterized by cell rounding were observed in secondary fetal brain cells and fibroblasts at about 70 MPa, whereas in the neoplastic neurogenic cell lines tested similar changes occurred at around 100 MPa, again demonstrating that malignant cells may be more resistant to HHP than their normal counterparts. Similar findings were reported by Yamaguchi *et al.* for Ehrlich ascites tumor cells demonstrating that these tumor cells stopped *in vivo* proliferation at HHP above 130 MPa (35).

#### 4. Effect of pressure on extracellular matrix proteins and enzymes

Little is known on the change of biological functions of proteins or other constituents of bone, cartilage, or tendon after exposure to HHP. Our own studies have demonstrated that the extracellular matrix proteins fibronectin, vitronectin and collagen-I present in the bone matrix have not deteriorated after HHP-treatment up to 600 MPa (10 min, room



temperature) with respect to cell proliferation, spreading and adherence of human osteoblast-like cells and human osteosarcoma cells (Saos-2) (11). These data encourage further exploration of the potential of HHP to sterilize tumor-affected bone segments prior to re-implantation, since during such treatment eukaryotic bone cells including tumor cells would be irreversibly impaired, while the bone's biomechanical properties and the biological properties of the extracellular matrix proteins fibronectin, vitronectin, and collagen-I would be preserved (11).

HHP causes a stress response in many types of mammalian cells, including chondrocytes and bone tumor cells (36). Further to this, Kopakkala-Tani *et al.* investigated whether some of the well known transduction pathways are activated in human chondrosarcoma cells under stress by exposure to moderate HHP of 15-30 MPa and demonstrated an increased level of active, phosphorylated forms of the extracellular signal-related kinase ERK and phosphoinositide 3-kinase under these pressure conditions (37).

HHP may not only exert an effect on tumor and normal cells present in the bone, but also on the tumor-associated proteases released by these cells, which are conducive to tumor bone turnover. At a pressure level of 600 MPa the latent activity of the inactive zymogens prothrombin, plasminogen, pro-uPA and trypsinogen, in addition to the proteolytically active forms thrombin, plasmin, HMW-uPA, and trypsin was minimally affected by HHP (24). The variation seen between different enzymes is probably due to differences in molecular structures and the resulting modifications after HHP treatment (24). It is worthwhile to note that at this pressure level normal bone cells and tumor cells are irreversibly impaired. Additionally, HHP also influences the activity of other enzymes. With that in mind, Masson *et al.* reviewed HHP technology and its potential applications in medicine and pharmaceutical science (9). The authors explained that HHP may affect both the activity and specificity of enzymes and that HHP is used for the engineering of proteins to allow enzyme-catalyzed synthesis of fine chemicals and pharmaceuticals and the production of modified proteins of medical or pharmaceutical interest. Such reactions can be used for food functionalization and for producing "nutraceuticals" to be used in complementary therapy (38). Pressure processing was found to be efficient in reducing the allergenic activity of food (39).

In general, pressures above 300 MPa cause irreversible protein denaturation at room temperature, whereas lower pressures may result in reversible changes in protein structure. The effects of HHP on enzymes have been divided into two classes: moderate pressure values of 100–200 MPa which activate monomeric enzymes and elevated pressures usually inducing enzyme inactivation (1). Investigations of the impact of moderate HHP up to 200 MPa on alpha-amylase have shown a pressure-dependent stabilization of the enzyme against temperature-induced inactivation (3,40). Interestingly, for some proteases, proteolysis enhancement through HHP (up to 400 MPa) depended on substrate changes and not on changes of the enzyme, as investigated for chymotrypsin in the hydrolysis of beta-lactoglobulin (41).

## 5. Effect of HHP-treatment on viability of microorganisms in bone

So far, the effect of neoantigens generated during HHP-treatment of bone, cartilage and tendon on the host after re-implantation has not been elucidated and is at present subject to

preclinical animal experiments. In spite of that, such physically modified proteins may be new innovative tools in the development of vaccines by making use of the changed immunogenicity of pressure-treated proteins or pressure-killed bacteria, viruses or normal and tumor cells (39,42-44).

Also of importance for HHP-treatment of bone is the fact that viruses are very sensitive to HHP, being inactivated at pressures as low as 100 to 300 MPa. Inactivation of numerous viruses such as herpes viruses, rotaviruses, influenza, picornaviruses as well as immunodeficiency viruses by pressure treatment has been successful in blood (45,46). The use of high pressure in decreasing virus concentration in the blood of patients suffering severe virus infections by *ex vivo* pressure treatment of blood has been proposed (47), but studies on HHP inactivation of viruses present in bone, cartilage or tendon have not been reported yet.

Likewise, different procedures are available to inactivate bacteria and fungi, including their spores, in human bone transplants (48). The most efficient methods of inactivation are gamma irradiation and thermal inactivation as well as chemical sterilization methods such as the peracetic acid-ethanol treatment of bone (49). The direct effect of HHP to achieve killing of vegetative bacterial, yeast and mould cells, has been documented as well (50,51), although much higher pressure values of 500 – 700 MPa are needed than for the inactivation of viruses (52,53). Interestingly, Gram-positive bacteria are more resistant to HHP than Gram-negative bacteria (54). A major advantage of HHP processing over gamma irradiation, thermal inactivation or the use of peracetic acid-ethanol treatment is that it preserves the initial mechanical properties of the bone, cartilage and tendon, a prerequisite for re-implantation of the *ex vivo*-treated tissues.

## 6. Conclusion

HHP technology has found broad application in the food industry, for instance in activating vegetative microorganisms in meat products, milk, juice, etc.

While viruses and bacteria can be inactivated by moderate to high HHP, outgrowth of tumor cells from tumor-afflicted bone and cartilage segments can be efficiently blocked by extracorporeal HHP, while leaving their biomechanical and key biological properties intact.

These findings raise the hope that HHP can eventually be used in orthopaedic surgery as an alternative technique over other established physical or chemical methods of sterilizing resected bone, cartilage or tendon in order to kill viruses, bacteria and cancer cells to allow autologous re-implantation. Still, before that goal is reached, further pre-clinical studies are required.

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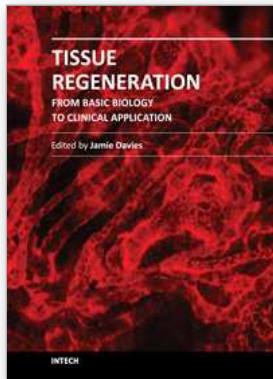
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When most types of human tissue are damaged, they repair themselves by forming a scar - a mechanically strong 'patch' that restores structural integrity to the tissue without restoring physiological function. Much better, for a patient, would be like-for-like replacement of damaged tissue with something functionally equivalent: there is currently an intense international research effort focused on this goal. This timely book addresses key topics in tissue regeneration in a sequence of linked chapters, each written by world experts; understanding normal healing; sources of, and methods of using, stem cells; construction and use of scaffolds; and modelling and assessment of regeneration. The book is intended for an audience consisting of advanced students, and research and medical professionals.

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