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Experimental Cryosurgery Investigations *In Vivo*

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Abstract

Cryosurgery is the use of freezing temperatures to elicit an ablative response in a targeted tissue. This review provides a global overview of experimentation *in vivo* which has been the basis of advancement of this widely applied therapeutic option. The cellular and tissue-related events that underlie the mechanisms of destruction, including direct cell injury (cryolysis), vascular stasis, apoptosis and necrosis, are described and are related to the optimal methods of technique of freezing to achieve efficacious therapy. *In vivo* experiments with major organs, including wound healing, the putative immunological response following thawing, and the use of cryoadjunctive strategies to enhance cancer cell sensitivity to freezing, are described.

Keywords

Cryosurgery; Cryoablation; Cryotherapy

Introduction

Cryosurgery is the use of freezing temperature to elicit a specific reactive response in tissue. The nature of the response depends upon the severity of the freezing injury. Minor freezing injury causes an inflammatory response, but severe freezing kills cells and results in destruction of tissue. An understanding of the mechanisms of cell and tissue injury due to freezing is only about sixty years in development. Though experience with cold injury and therapy is described in ancient manuscripts and in experiments with frostbite in the last 19th century, most current knowledge is based on investigations that began in the 1940's, initially with frostbite and then with the use of freezing techniques to preserve cells or produce local lesions for physiological investigations. By early in the 1960's, experimental studies had shown that cell and tissue death from freezing was due to direct cell injury and to the vascular stasis that developed shortly after the thawing period. The relative importance of these two mechanisms of injury was a matter for debate. Investigators of frostbite were impressed by vascular stasis produced by freezing, but investigators of cryopreservation focused on ice crystal formulation and its

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deleterious effects. Nevertheless clearly both mechanisms are operative in freezing injury of any type or cause.

Experiments pertinent to cryosurgery on local tissue injury by freezing, which also began about sixty years ago, produce lesions which resemble frostbite. Investigations *in vitro* and *in vivo* have shown that the major mechanisms of injury are due to direct cell damage, which begins as tissues falls into hypothermia, and the failure of the microcirculation which develops after thawing [60]. The considerable research, which has been done *in vitro* has provided detailed information on the effects of freezing on cells. But substantial differences may exist between the effects of freezing cells *in vitro* versus freezing *in vivo*. The *in vitro* work, performed in the absence of the vascular factor of injury, permits close examination of the effect of the diverse facets of the freezing and thawing cycle. Whether directed at cell preservation or destruction, the *in vitro* work is of substantial importance and recently has clarified the molecular-based mechanisms in the complex cell death cascade [11]. Nevertheless, in cryosurgery, the effect of freezing on the vasculature is clearly a critical and perhaps dominant cause of cell death. With thawing, the microcirculatory failure in the previously frozen tissue is progressive, resulting in vascular stasis in about one hour, which insures cell death from ischemia. Therefore research *in vivo* by cryosurgical techniques is critical to knowledge of freezing for therapy.

The range of investigations *in vivo* is substantial and covers every facet of cryosurgery. Many experiments focus on the technical features of cryosurgery, that is, the manipulation of the freeze-thaw cycle to enhance destruction or to achieve a cell-selective response. Other work investigates the monitoring of the freezing process in the tissue, principally by imaging, but also by other techniques. Many investigations detail the effects of freezing tissue in terms of wound healing which varies with the structure of the tissue. Recent research has investigated adjunctive therapy with cytotoxic drugs, chemical agents or irradiation as a means of enhancing the efficacy of cryosurgical treatment. Though experiments *in vitro* and clinical results are mentioned occasionally to support or verify the work in animals, this review focuses on the diverse aspects of cryosurgical research *in vivo*.

Direct Cell Injury

The cornerstone of direct cell injury from freezing is ice crystal formation which removes water from the cells and leads to a sequence of deleterious events [126,132]. Recent investigations *in vitro* have identified apoptosis, or gene regulated cell death, as a mechanism of direct cell injury [89]. This was confirmed by experiments *in vivo* by Steinbach *et al*, showing that cell death is by necrosis in the central part of the cryogenic lesion and that apoptosis is evident in the peripheral part of the lesion, generally apparent in 8 to 12 hours [195]. Recently the investigations of Forest *et al*. used a human lung adenocarcinoma cell line injected into the dorsal region of mice and showed that the central part of the frozen volume was necrotic, but the peripheral zone had apoptotic cells. Apoptosis increased progressively in the period from 2 hours to 8 hours after freezing. Apoptosis occurred in the zone beyond the necrotic area, though the borders between the two were not clear. A second peak of necrosis was observed after four days [54,55]. Wen *et al*, experimenting with freezing lung adenocarcinoma transplanted subcutaneously in mice, noted necrosis as the method of cell death in the central part of the tumor, peaking at 68% of the volume in four days. In the peripheral zone of the lesion, they noted apoptotic cells, peaking 8 to 16 hours after freezing. The apoptotic change was thought to be operative through mitochondrial damage due to increased bax protein expression and caspase activation [210]. These several experiments *in vivo* confirm the importance of apoptosis as a mechanism of cell death after cryosurgery especially in the periphery of the previously frozen tissue and at the same time illustrate a direct link between *in vitro* and *in vivo* studies.

The Vascular Injury

Early in modern cryosurgery, many experiments with diverse tissues, such as rat liver, guinea pig skin, rabbit skin, and hamster cheek pouch, showed the sequence of events that follow freezing and thawing and ending in circulatory failure. When the tissue thaws, the previously frozen volume of tissue becomes congested and edematous, which extends progressively during the next few hours. The cause of the edema is endothelial cell damage, manifest as defects in endothelial cell junctions as early as two hours after thawing, resulting in increased capillary permeability, edema, aggregation of platelets, and finally thrombosis. The loss of blood supply results in necrosis except at the periphery of the previously frozen tissue where the tissue temperature is in the range of 0 to -20°C degrees. In this border zone, though many cells will die, some cells will survive. The relative importance of direct cell injury and failure of the microcirculation remains unclear, though both are operative in cryosurgery [60,86].

Technology-Methods and Control of Freezing

A large variety of cryogenic agents and freezing techniques with diverse apparatus have been used in cryosurgical experiments and treatment. Investigators in the first half of the 1900's produced lesions by freezing various tissues and developed a considerable amount of knowledge pertinent to cryosurgery. These experiments, which defined the cryosurgical lesion, helped pave the way for the modern era of cryosurgery, initiated by Cooper and Lee in 1961 when they developed automated apparatus cooled by liquid nitrogen (-196°C) for application to the tissue [39]. Years later, the development of apparatus which cooled multiple probes simultaneously or in sequence with super-cooled liquid nitrogen improved the clinical versatility of the cryosurgical techniques [10,26]. In recent years, argon (-186°C) and nitrous oxide (-89.5°C), which had limited use as cryogenic agents in the past, came into common use in apparatus as pressurized gases, cooling by the Joule-Thomson (J-T) effect. The use of pressurized gases, especially argon and nitrous oxide, permitted the construction of diverse instruments, including thin needle-like probes, catheter probes, balloon structures, and clamp devices.

These modern devices, cooled by diverse cryogens, had some differences in freezing capability, which were related principally to the cryogen in use. The volume of tissue frozen in a single application of a cryoprobe is directly related to the temperature of the probe, the area of contact with the tissue, and the duration of contact. The thermal conductivity of the tissue is also a factor. To use two or more probes simultaneously introduces a measure of uncertainty in appropriate placement and the extent of interactions during freezing. To integrate the freezing actions of multiple probes with the dimensions of the target tissue, computerized planning of cryosurgical treatment merits a large measure of attention [165].

Numerous experiments have focused on the methods of monitoring the progress of tissue freezing. Needle-thermocouples to measure the tissue temperature at appropriate sites came into common use early in the modern era of cryosurgery, but these did not provide a global view of the frozen volume. The use of imaging techniques, beginning with the demonstrations of Onik *et al.* late in the 1980's, provided a method of monitoring the process of freezing by ultrasound [151,152]. This led to the renaissance of interest in cryosurgery in the 1990's. Ultrasound is the common method of monitoring the process of freezing, but ultrasound has limitations from acoustic shadowing [117,209]. Its use can and often should be combined with the use of thermocouples at appropriate places in the tissue.

Investigations on the practicality of computerized tomography (CT) and magnetic resonance (MR) imaging to monitor the freezing of tissue quickly followed [179,197,199]. Computerized tomography has the advantage of providing a three-dimensional image. MR imaging provides a three-dimensional image and, with appropriate modeling software can predict the isotherms

in the frozen tissue. A close correlation between these image monitoring techniques and the volume of the cryolesion during and after freezing has been demonstrated in experiments. The volume of destroyed tissue was slightly less than the frozen volume which is to be expected because the cells in the tissue temperature range of 0 to -20°C are not completely destroyed. Other less commonly used monitoring techniques of experimental interest include thermography and impedance measurements, including the recently evolving technique of electrical impedance tomography [48,113,154,170].

The Freeze-Thaw Cycle

An understanding of the mechanisms of cell and tissue injury requires recognition of the effects of the freeze-thaw cycle as used in cryosurgery. Every facet of the freeze-thaw cycle may produce injury to the tissue, and all may be manipulated. Therefore knowledge of the effect of each phase of the cycle is critical, whether the goal is complete or selective tissue destruction. Though the components of the freeze-thaw cycle are difficult to describe with precision in a frozen volume of tissue, their individual role in cryogenic injury have been evaluated in many experiments.

The Cooling Rate

Experiments have shown that intracellular ice crystals, considered lethal for cells, form over a wide range of cooling rates, including at slow rates [15,87]. This is of importance to freezing tissue *in vivo* because much of the frozen volume of tissues is subject only to slow cooling rates, that is, about $10^{\circ}\text{C}/\text{minute}$ or slower. Though slow cooling tends to produce extracellular ice, these large crystals, considered rather harmless in cell suspensions, may be lethal when occurring in tissues with closely packed cells. Only the tissue close to the heat exchange surface of the cryoprobe is frozen rapidly. The cooling rate at more distant sites slows as the volume of frozen tissue expands. In about 10-15 minutes, an equilibrium between heat loss and heat gained from blood supply is realized and little more expansion is possible to achieve depth of freezing or increased volume. Diverse types of tissue have different thermal characteristics, which modify the volume of tissue frozen by cryoprobe [158]. Experiments *in vivo* have shown that the cooling rate; whether slow or rapid, is not as important as other factors in the freeze-thaw cycle [65].

The Tissue Temperature

The tissue temperature is a key factor in causing injury. Experiments *in vivo* show that the vascular stasis after thawing modifies the interpretation of the lethal temperature for cells and have introduced a measure of uncertainty about appropriate temperature goals in cryosurgery. As a guide for the treatment of neoplasms, the many experiments suggesting that about -20°C is adequate for tissue destruction should be viewed with caution. Certainly extensive tissue damage occurs in the -20 to -30°C range, but tumor cell destruction at the temperature range is uncertain and incomplete. Neel *et al.* experiments with animal tumors, set the required temperature for tumor destruction at -60°C , and stated the need for repeated freezing in addition [146]. In experiments with diverse animal tissues, investigators have set the lethal temperature in the -40 to -50°C range. A variety of opinions on the lethal temperature, or in other words, the appropriate temperature goal in cancer cryosurgery, persist to this day (Table 1). The reason for the differences in opinion lies in the use of different cells and tissues in varying experimental conditions. The resistance of cancer cells shows the need for repeated freeze-thaw cycles. A reasonable view is that cells frozen to temperature colder than -40°C are likely to be destroyed by direct cell damage, but vascular stasis will have a wider destructive effect over the warmer freezing temperatures.

Duration of Freezing

The optimal duration of freezing, that is, how long the tissue should be in the frozen state, is not well established, but experiments have shown that prolongation of freezing produces a greater destructive effect [19,65,194]. Nevertheless in general, the duration of freezing is unimportant if the tissue is held at temperatures colder than -50°C . However, holding the tissue for longer periods at -10 to -25°C range will increase destruction because of solute effects and recrystallization [127].

The Thawing Rate

Slow thawing of the frozen tissue is a prime destructive factor. Experience with frostbite and with cryopreservation has shown that rapid warming increases the chance of cell survival. The longer the duration of the thaw, the greater the damage to the cells because of an increase in solute effects and maximal growth of ice crystals. The large ice crystals create shearing forces which disrupt the tissue. Experiments, using tissue *in vivo*, have shown deleterious effect of slow thawing [65,143]. Whittaker showed that intracellular ice crystals are larger in the second freezing cycle and suggests that this effect was due to a longer thaw time [213]. Judging from frostbite experience and experiments *in vivo*, the thawing rates should be as slow as possible, that is, determined by the input of body heat into the frozen lesion.

Repetition of the Freeze-Thaw Cycle

Since the beginning of modern cryosurgery, the initial clinical reports in 1965 stressed the need for repetitive freezing in the technique of cancer cryosurgery [22,38,67]. The difficulty in destroying cancers by freezing was obvious in that early experience. The second cycle produced faster and more extensive tissue cooling, so that the volume of frozen tissue was enlarged and the border of certain tissue destruction was moved closer to the outer limit of the frozen volume.

Experimental evidence confirming the increased destructive effect of the second cycle is substantial (Table 2). The enhanced lethal effect of repeated freeze-thaw cycles is best noted in the elevated freezing temperatures, that is, tissue temperatures in the -20 to -30°C range. Of course it is in this range that the increased destructive effect is most needed. At tissue temperatures of -40 to -50°C and cooler, the temperature is sufficiently low to destroy the tissue in a single cycle. The major reason to use a repeated freeze-thaw cycle is to extend the lethal effect into the warmer freezing temperature zone at the periphery of the defined target tissue. The evidence supporting the use of repeated freeze-thaw cycle is so strong that most clinicians who use cryosurgery for cancer have adopted this facet of the technique.

The Interval Between Freeze-Thaw Cycles

The interval between freeze-thaw cycles is a factor of importance in tissue injury, but rather little attention has been given to this phase of the freeze-thaw cycle. Whittaker, in experiments using repeat freeze cycles in the oral mucosa of the hamster, has shown that intracellular ice crystals are larger with increasing time between freezes [213]. Therefore he suggested that the longer thaw time of the interval will increase the efficacy of the technique. A longer thaw time allows for ice recrystallization and osmotic damage. Then a little further wait, leaving the tissue in the hypothermic state, provides time for the microcirculation to fail. The heat brought to the area lessens and the second freezing cycle is more effective. In clinical practice, the urge and the need to move on with the procedure is so great that many physicians will not have the patience to wait for the completion of full thawing or a prolonged interval between cycles.

In summary, optimal technique for the destruction of tumors is fast freezing of the tissue to an appropriate low temperature, slow thawing, (which increases the duration), and repetition of the freeze-thaw cycle. Selective cell destruction requires modification of the freezing program.

The Cryogenic Lesion

Before the modern era began in the 1960's, local freezing techniques were used to destroy tissue, as in the brain, heart, liver, and kidney of rabbits and cats, for physiological studies [58]. These experiments accurately described the nature of the cryogenic lesion which investigators characterized as a volume of circumscribed necrosis, featuring a large central necrotic region surrounded by a narrow peripheral or border zone of partially damaged and surviving cells. The peripheral or border zone of the cryogenic lesion is a region of great interest because of adjunctive therapeutic implications. In this region, the cooling rate is slow, the duration of freezing is short, the final temperature is in the range of 0 to -10°C , and warming is rather rapid. In this zone, some cells are necrotic, others are apoptotic, and others may survive. Apoptotic cells will become necrotic under the anoxic conditions typical of the cryogenic lesion [11,12,52,95,171]. The chance of cell survival close to the 0°C isotherm is good because many cells can supercool to -10°C or even -15°C and the osmotic and hypothermic stresses are often insufficient to cause cell death [126,127,132]. It in this range that differences in cell sensitivity to freezing injury become evident.

Cell Sensitivity to Freezing

Diverse types of cells have different sensitivities to freezing temperatures. These differences can be identified in single, short freezing cycles. Experiments show that the temperatures at which the differences in cell sensitivity can be identified are in the range of 0 to -30°C [59, 88]. Sebaceous glands and hair follicles were also lost in this warm freezing range, but keratinocytes survived at -30°C . Experiments with freezing the canine femur have shown that osteocytes are killed at about -10°C [64]. Normal cells of the kidney, liver and prostate die at about -15 to -20°C [170,177,185,191,192]. Fibroblasts resist freezing injury. Tumor cells show their variable and extraordinary resistance to freezing injury.

Apart from the sensitivity of the cells to freezing injury, the structure of the tissue is important. Since fibroblasts and collagen fibers resist freezing injury, the architecture of the tissue, though devitalized, remains as a structure for repair [62,115,141,187]. Large blood vessels continue to function as conduits of blood. Bone appears unchanged after devitalization by freezing. The structure of nerves remains intact. The preservation of tissue structure is critically important to wound healing.

Wound Healing

The healing of the cryogenic wound is related to the severity of the freezing injury and the type of tissue. Minor freezing injury, such as that produced by short exposure to a temperature about -10°C , is likely to result in little tissue loss and will heal quickly. At a tissue temperature of -20 to -30°C , tissue loss will be greater and directly related to the temperature and duration of freezing. Repair of the cryogenic wound is usually favorable. Though the tissues are devitalized by freezing, the matrix may be little changed and the preservation of this architecture is important to repair. Wound healing is an active process that begins with an inflammatory reaction at the border of the lesion, induced by chemotactic factors. Early infiltration of neutrophils, then mononuclear cells, is stimulated by the mediators of inflammation, including prostaglandins, histamine, and cytokines. This cellular infiltration follows hypothermia and edema that develops with thawing of the frozen tissue. Investigators have suggested that the inflammatory cell infiltrates contribute to the development of apoptosis and to tissue destruction [70,181]. As a granulation tissue forms, fibroblasts differentiate into myofibroblasts and damaged collagen is replaced by new collagen [70,99,181]. The cellular infiltration helps establish the new vasculature, which is critical to the repair of the repair process of the devitalized tissue [57,64]. Delay in healing is characteristic of cryogenic wounds.

Time is required to clear the necrotic tissue, whether by slough or by resorption, so healing is slow in comparison to excision and primary suture of a wound.

Diverse Tissue

The freezing of most tissues produces the typical cryogenic lesion of demarcated coagulation necrosis. The process of healing, though similar in cellular infiltrations and establishment of new blood supply, differs in the diverse tissues. The skin is high in collagen, elastin, and fibroblasts, which resist freezing injury, so healing is ordinarily favorable. Damaged collagen is absorbed and slowly replaced by new collagen. Muscle fibers and cellular tissue, such as the liver and kidney, heal with replacement by fibrous tissue, which may be a lengthy process, depending upon the volume of tissue frozen. The architecture of nerves, major blood vessels, and bone is largely preserved after devitalization and serves as a scaffold for repair. Diverse tissues and organs have been the subject of experiments preliminary to clinical application of cryosurgery, commonly for the treatment of tumors. These tissues have been the focus of considerable research *in vivo*. Use of cryosurgery in some tissues is common in clinical practice and has little need for further experimental animal work. Experiments on some tissues were performed early in cryosurgery and have not been continued because no clinical application is envisioned.

The Skin

The effects of freezing the skin were described in clinical observation early in the 1900's [58]. Physicians of that era were impressed by the good healing and lack of scarring after freezing skin lesions. Experiments with skin have confirmed this view. In experiments in rats, Li *et al.* produced full thickness wounds by hot thermal burns and by freezing for the purpose of comparing the difference in healing [115]. The degree of tissue destruction by the two injuries was similar. The burn wound contained much more collagen than the freeze wound. The authors thought that slow removal and replacement of the collagen prevented the development of a contracted scar. A later study in rats by Ehrlich and Hembry again showed that the connective tissue matrix was important in controlling wound contraction [50]. Shepherd and Dawber, observing the effect of freezing the skin of pigs, noted that fibroblasts were resistant to freezing [187]. No fractures or dislocation of collagen fibrils were seen. Long-term observation showed some thinning of the skin. Experiments by other investigators showed the sensitivity of melanocytes, hair follicles, and skin glands to mild freezing temperature [68,141]. The resistance of collagen fibers in skin to damage from freezing is the basis for favorable healing. These diverse experiments provide information consistent with the vast clinical experience with the use of cryosurgery for skin disease over many years.

The Liver

Some early experiments *in vivo* with the liver were directed at the possibility that freezing might be useful as a hemostatic agent [53,186]. This benefit was never achieved. Tissue does not bleed while frozen, but on thawing, some bleeding occurs. Recent work by Kopelman *et al.* examining freezing as a method of controlling the bleeding from crush injuries to the liver of pigs, showed that freezing is not hemostatic for major vessels, but it might be useful in the microcirculatory vessels to help control oozing of blood [107].

The major interest in liver cryosurgery quickly became focused on the treatment of tumors. The typical cryogenic lesion in the liver, consisting of a demarcated volume of coagulation necrosis with a narrow border of partially damaged tissue, healed by inflammatory cell infiltration neovascularization and fibrous tissue replacement, in time yielding a fibrous contracted scar. Freezing is potentiated by occluding the blood supply to the liver [147]. With clinical application to large liver tumors in mind, Dutta and his associates focused on large

volume freezing of the liver in dogs, using aggressive freezing techniques with cryoprobe and by pouring liquid nitrogen into a funnel on the liver surface [46,47]. Cracking of the liver surface, causing bleeding, was a problem, which they solved by slowing the rate of cooling by placing a thin felt layer between the probe and the liver.

Early in the modern era, the development of the cryosurgery of liver was hampered by a lack of adequate methods of detection of deep lesions. In the mid 1980's, Onik and his colleagues demonstrated that ultrasound could be used to monitor the process of freezing the liver, providing an image as the frozen tissue expanded in volume [152]. Interest in experimental and clinical use quickly grew and techniques evolved. Seifert *et al.* freezing the liver in rats, noted that large volume freezing was associated with cytokine release, which can be deleterious [184]. Nevertheless, experimental applications and extensive clinical use have shown that cryosurgery of the liver is safe and efficacious.

The Kidney

The kidney responds to local freezing in the same manner as other visceral organs, that is, with creation of a circumscribed volume of coagulation necrosis [24,190]. The shapely demarcated lesions showed a mild inflammatory response and late fibrous scar formation. The lethal tissue temperature for normal kidney cells has been reported to be about -20°C in single freeze-thaw cycles [29,41]. Wooley *et al.* in experiments in dogs, noted the greater destruction produced by repeater freezing cycles [214]. Collyer *et al.* working with pigs, demonstrated that enhanced size of the frozen volume and necrosis was achieved with intra-renal cooling with saline-ice slush infused into the renal pelvis during laparoscopic cryoablation [36]. To resolve concerns about the effect of freezing on the renal pelvis, Janzen *et al.* in experiments with swine, showed that the collecting system was preserved [97]. Other investigators have produced freezing within the renal pelvis in pigs and noted no urinary fistulas or urinary extravasation [17,188, 196].

In summary, extensive experimentation has demonstrated the safety of local freezing of the kidney. The previously frozen tissue heals and slowly contracts in size as fibrous tissue replacement progresses. These experiments provide a solid basis for the use of cryosurgery to ablate small tumors of the kidney.

The Prostate

Cryosurgery of the prostate began with the experiments of Gonder and his associates who reported on the freezing of the prostate in dogs in 1964 [75]. Numerous clinical trials continued through the 1970's, but prostatic cryosurgery fell into disuse early in the 1980's [66]. Then Onik and his associates described the use of ultrasound to monitor the process of freezing [151]. This quickly regenerated interest in prostatic cryosurgery. Littrup and his associates investigated the relationship between the ultrasound image and the later histologic changes in freezing the canine prostate [117]. The correlation between image and tissue necrosis was excellent, that is, necrosis developed immediately behind the echogenic leading edge of ice formation. Residual outlines of ducts and acini could be recognized and these served as the architectural skeleton for repair. In three weeks, transitional cell ingrowth and glandular formations were seen. The stroma had many fibroblasts.

During prostatic cryosurgery, to reduce complications, the urethra needs protection. Cohen and Miller devised a warming catheter for thermal protection, which quickly became clinically useful [34]. With the thought of protecting the nerves against freezing, Janzen *et al.* investigated the effect of active warming of the neurovascular bundle after freezing the prostate in dogs [96]. The bundles were warmed with helium gas while the prostate was frozen in single or double cycles. They reported that preservation of the neurovascular bundle was possible but

not consistently reproducible. In dogs, the warming of the bundle resulted in incomplete peripheral prostatic tissue ablation. Nevertheless, clinical interest in nerve-sparing focal therapy is evident [153]. Recently Cohen *et al.* reported on their 10-year retrospective experience with prostate cryoablation [35]. In 2008 the American Urology Association published a best practices policy statement which recommended prostate cryoablation as both a primary and secondary therapy [6].

The Pancreas

The pancreas has been frozen with cryosurgical techniques in diverse animals, including dogs, pigs, hamsters, and monkeys. In general, most reports state that limited pancreatic freezing can be done without mortality and that the effect features an inflammatory response, including a sharp rise in serum pancreatic enzyme levels for several days, followed by progressive changes leading to necrosis. Korpan described the ultrastructural changes following pancreatic freezing in considerable detail with an emphasis on vascular changes [108]. Healing features fibrous tissue replacement and gradual wound contracture, just as in other organs [140].

Freezing a larger volume of the pancreas may be less well tolerated. McIntosh and his associates, freezing 50% of the pancreas in dogs, caused acute hemorrhagic pancreatitis in all animals with 38% mortality rate [130]. None of the surviving animals developed functional insufficiency of the gland. In later experiments, they froze the pancreas and portal vein simultaneously in pigs with a liquid nitrogen probe to a temperature of -100°C , measured at the interface between probe and tissue. The portal vein wall showed some fragmentation of fibers and was thickened. No mortality resulted [129].

In summary, limited pancreatic freezing is safe. Extensive freezing may result in problems related to loss of pancreatic function. Little clinical use has followed this experimentation, except in Russia where clinical reports describe use for pancreatitis.

The Breast

Breast cancer was first treated in the mid 1850's by Arnott, a physician in England, who used iced saline solution to freeze advanced accessible cancer and achieved palliative benefits [58]. Only in recent years has some experimental work been performed in animals. Rand *et al.* used a virus-induced mammary adenocarcinoma in mice to examine the effect of freezing *in situ* [167]. Freezing was continued until the ice front extended several millimeters beyond the tumor. Repeated freezing was needed to prevent recurrence. Staren *et al.* in experiments with freezing mammary adenocarcinoma in rats and mice with a 3 mm probe cooled by liquid nitrogen, found that -40°C at the periphery of the tumor was not sufficient to destroy tumors 1.5 to 2.5 cm in diameter [194]. An enhanced destructive effect was achieved by prolonging freezing to 15 minutes and repetition of the freeze-thaw cycle. Rabin *et al.* examining the long term healing after producing cryogenic lesions in the sheep breast by freezing with a novel device cooled by liquid nitrogen in single or multiple freezing cycles, then following the progress of the lesion with ultrasound, noted healing by collagen deposition, yielding finally a contracted scar [166]. In 5 months after freezing, the injured tissue was about one-half the size of the imaged frozen region. The border zone had damaged lobules without acini. Otterson *et al.* produced lesions in the breast of the goat with a probe cooled by liquid nitrogen to about -160°C [155]. Two freeze-thaw cycles, each 3 minutes in duration were used. Tissue temperature was not recorded. Skin freezing was minimized, but depigmentation was noted in dark-skinned goats. Healing progressed in the usual manner, that is, cellular infiltration, revascularization, and fibrous tissue replacement that developed into a fibrous scar by 7 weeks after freezing.

In summary, freezing the breast produces typical cryogenic lesions. Healing is satisfactory and results in a contracted fibrotic scar. This experimental work has provided a basis for the clinical trials for tumor therapy in recent years.

The Esophagus

Experience with esophageal freezing has been directed at potential clinical application to esophageal disease and also prevention of injury when freezing adjacent structures, especially the heart. Rodgers *et al.* cooled the distal esophagus of cats to -70 to -80°C for two minutes with a cryoprobe [172]. Superficial ulcerations were produced, but no loss of esophageal function resulted. More profound freezing produce deeper injury and esophageal perforation [173].

Endoscopic freezing of the esophagus was reported by Pasricha *et al.* who sprayed the esophageal mucosa of dogs with liquid nitrogen for 2 to 4 seconds [156]. Superficial mucosal necrosis was produced, which healed with cellular infiltration and re-epithelialization. Similar endoscopic experiments were done by Johnston *et al.* who sprayed the distal esophagus of pigs with liquid nitrogen for 10 to 60 seconds until the mucosa appeared white and frosted [100]. As the injury developed, superficial erosions about 2mm deep, extending into the submucosa, were formed. Circumferential freezing, which had been done in 3 pigs, resulted in esophageal stricture.

Since esophageal injury has been reported after freezing the pulmonary veins in the treatment of atrial fibrillation, some experiments have been directed at freezing the esophagus from its external surface. Freezing from within the cardiac atria and pulmonary veins in sheep, using probe temperatures of -160°C for two minutes, Aupperle *et al.* and Doll *et al.* freezing the external surface of the esophagus in sheep, noted injury to the muscular layer, but the epithelium was not involved [2,43]. Ripley and his associates froze the cervical esophagus of calves with a 6.5mm catheter probe at a temperature of -80°C for a five minute cycle [169]. Full thickness necrosis was not produced.

In summary, the sensitivity of esophageal tissue, especially the mucosa, to freezing has been shown. The response may prove useful in the treatment of dysplastic disease. Esophageal injury following cryoablation in the pulmonary veins may occur, but is not likely to be transmural.

Trachea-bronchus-lung

Experiments in the 1970's demonstrated that trachea, bronchus, and lung can be safely subjected to local freezing [144,202]. All layers of the trachea and bronchi were destroyed. Necrosis was followed by healing with prompt regeneration of the mucosa and early regeneration of the epichondrial layer of cartilage. Neel and his associates froze the lung via thoracotomy in dogs and monkeys, compressing the tissue against the freezing probe without penetrating the lung [145]. After thawing, the lesion appeared as a hemorrhagic infraction. Healing yielded a contracted fibrous scar. Recently Izumi *et al* investigated the feasibility of transthoracic intubation of a cryoprobe into the parenchyma of the lung [94]. Using pigs, a 2mm diameter probe was inserted 3cm into the lung and one or two cycles of freezing, 15 minutes in duration, were performed. The use of two cycles increased the bleeding time and decreased the leakage of air.

In summary, freezing the lung presents the risk of air leak if the serosal surface is penetrated. Since the lung contains air, freezing is hampered unless the lung is compressed. The early experiments were followed by a rather large clinical experience with freezing selected bronchial cancers via endoscopy [122].

Blood Vessels

The reaction of large arteries and veins to freezing *in situ* is detailed in reports describing experimental cryogenic injury to a variety of major blood vessels [40,63,69,111,123]. The large blood vessels were remarkably resistant to structural change after freezing and their function as a conduit of blood was not impaired. The injury featured endothelial loss and some disruption of the architecture of the arterial wall. Healing featured smooth muscle proliferation, myointimal thickening, and endothelial regeneration. During this time the function of the artery as a conduit of blood continued. The effect of freezing on the major coronary arteries is similar, but is of special interest because of their critical importance to cardiac function, so these arteries will be mentioned again in relation to the heart. Though the major arteries continue to function, in general, the smaller the blood vessel and the more severe the freezing injury, the more likely that late thrombosis will develop. On the microcirculatory level, thrombosis after freezing is certain to occur.

In most of the experiments already cited, large veins, such as the femoral vein, the portal vein, and the inferior vena cava were frozen. These studies, making allowance for the fact that large veins are mostly fibrous tissue and have little or no elastic or muscle tissue, showed no basic differences from the changes in the arteries. McIntosh and his associates, in experiments with freezing the portal vein in pigs, demonstrated the same characteristic intimal thickening and subintimal proliferation of fibrous tissue and the minimal changes in the structure of the media [129]. None of the veins were occluded by thrombi. Recent experiments have examined the effect of freezing the retrohepatic vena cava [49,102]. These experiments have shown that the vena cava, though damaged by freezing even to the extent of complete necrosis of the intima, went through repair without complications.

Experiments to control the smooth muscle proliferation which follows angioplasty, causing vascular stenosis, have involved freezing the injured vessel with a cryocatheter or balloon. Cheema *et al.* examined the effects of intravascular cryotherapy on arterial wall repair after balloon angioplasty in the iliac artery of rabbits [28]. They found that the freezing produced no benefit when compared to balloon angioplasty alone. The collagen accumulation in the three layers of the artery contributed to late inward remodeling of the balloon-injured artery. In these experiments, the average arterial wall temperature was -26°C , which may be too cold to achieve benefit in terms of a selective effect. Tanguay *et al.* using balloon angioplasty to produce vascular injury in the carotid artery in swine, followed by endoluminal freezing in several temperature protocols, found that freezing at -50°C increased the density of collagen [200]. Late luminal loss was prevented by improving the vascular remodeling. The benefit was attributed to the increased rate of synthesis of collagen. The arterial wall temperature was not cited.

In vitro experiments are particularly important to the question of control of smooth muscle proliferation. Hollister *et al.* subjected cultured human coronary artery endothelial cells and smooth muscle cells to a range of freezing temperature [89]. The experiments showed that the smooth muscle cells were more sensitive to freezing injury than the endothelial cells in the range of 0 to -15°C . The difference was small, but suggested that it might be possible to use a cooling temperature that would preserve endothelial cells and inhibit smooth muscle proliferation. In similar experiments, Tatsutani *et al.*, working with cultured arterial cells and smooth muscle cells, found that the smooth muscle cells were susceptible to freeze-induced apoptosis in the range of -5 to -15°C , which led to the conclusion that endovascular cryotherapy might limit neointimal formation and thereby reduce the incidence of restenosis [201]. Working with an *in vitro* tissue equivalent model, freezing smooth muscle cells, Grassl and Bischof reported a 30% survival at -11°C [77]. Yiu *et al.* reported on the sensitivity of vascular cells to temperatures of -10°C and described the increased apoptotic changes produced by a second super cooling cycle [215,216]. These diverse reports of work *in vitro*

are generally in agreement and show the need for experiments *in vivo*. To better define the range of temperature which will permit preservation of endothelial cells while inhibiting smooth muscle proliferation. As adjuncts to freezing, the use of pharmaceutical or genetic agents may facilitate the process.

The Heart

A large volume of experimentation detailing the effects of freezing on the muscle, valves, blood vessels, and conduction system of the heart is available for review. Cardiac muscle is sensitive to freezing injury and heals with fibrous tissue replacement of the devitalized tissue [82]. When the potential for clinical application of freezing techniques to heart rhythm disorders was recognized and as new cryosurgical apparatus was developed, experimentation was stimulated. Many experiments described again the cryogenic lesion described by Haas and Taylor years earlier. These experiments, often investigating the electrophysiological effects, have shown the feasibility of creating ventricular lesions by freezing without causing serious cardiac malfunction [72,105,106,121,136]. Even the papillary muscles of the left ventricle have been frozen without causing serious consequences in relation to mitral valve function [9,78].

Experiments, freezing the left ventricle in animals, have shown the difficulty in producing transmural lesions in the beating heart [133]. Lustgarten *et al* using a catheter structure with a 3 cm linear freezing surface on a 6mm tip, used at a probe temperature of -90°C for four minutes, could produce a ventricular lesion only 2.7 mm deep [120]. However, Wadhwa *et al.* were able to produce transmural lesions in the beating heart in the ventricle of dogs by freezing with a 10F cryoablation catheter at -79.6°C or colder [205]. Masroor *et al.* were able to produce transmural right ventricular lesions during epicardial freezing in pigs [125]. Kettering *et al.* produced long linear lesions with a cryocatheter in pigs by using multiple points of application at -75°C for four minutes [104]. Milla *et al.* used a novel clamping device to freeze the atria [134]. The target of these experiments was the conduction system of the heart, that is, the sinoatrial (SA) node, the atrioventricular (AV) node, or aberrant conducting myocytes.

Interest in cardiac cryosurgery centers on the treatment of disorders of the conduction system, principally the tachyarrhythmias. The conduction system, including the nodal structures and the cardiomyocytes, are sensitive to freezing. Harrison *et al* produced AV nodal block in dogs by freezing to -55 to -60°C contact temperature for about two minutes [81]. Other investigators have produced AV blocks in animals using diverse devices [73,104,105,205]. The AV node, if subjected to a minor cold injury (0 to -5°C), as produced by a probe applied at -30°C for 1-2 minutes, will temporarily lose function and a temporary heart block will result until the node is warmed. Partial damage and delayed recovery will occur in the -5 to -15°C range. Permanent AV nodal block will be produced by freezing with the probe at -70 to -75°C , achieving a tissue temperature of about -20°C in about 4 minutes. Dubuc *et al.* using a catheter cryoprobe, reported that a double cycle was needed [45]. Avitall *et al.* produced AV block in dogs by placing a balloon device, measuring 10-19mm in the coronary sinus and cooling the balloon to about -76°C for three minutes [3]. Transmural lesions across the AV groove were created.

Recently, as a mechanism of atrial fibrillation/flutter, the importance of specialized myocardial conduction fibers extending into the pulmonary veins from their junction with the right atrium has been shown [80]. Experiments have shown that these fibers can be destroyed by freezing, using diverse devices at temperatures of -65 to -80°C for about four minutes to produce lesions encircling the pulmonary veins [79,180]. Right phrenic nerve injury is a risk. Pulmonary vein stenosis is not a risk. Avitall *et al.* using cryoballoon technology placed in the pulmonary veins in dogs, successfully produced electrical isolation at the junction with the atrium, but most of the dogs had hemoptysis in the first 24 to 48 hours due to extension of freezing into the lung

[4]. The freezing may penetrate the outer layers of the esophagus, but transmural esophageal lesions were not produced.

In freezing the heart, concern with the effect of freezing the coronary arteries led to experiments on this subject. Mikat *et al.* used a probe about 5mm in diameter to freeze the bifurcation of the left anterior descending coronary, arresting blood flow. Two freeze-thaw cycles to -40 to -50°C for two to three minutes each were used. Thawing was slow between cycles. In a week after injury, the arteries showed medial and intimal alterations, including necrosis and presence of inflammatory cells. The internal elastic lamina was discontinuous with thickening of the intima, including the presence of smooth muscle cells. Endothelial erosion was present, which led to thrombosis in medium to small arteries in one week [133]. Misaki *et al.* freezing directly over the left coronary artery in sheep, found that only minimal arterial changes developed and that the vessel remained widely patent after freezing injury [136]. Similar results have been reported by others [90,92]. The experiments of Bakker *et al.* showed that the small epicardial arteries were subjected to thrombosis [8].

In summary, cardiac tissues are sensitive to freezing injury. Freezing the myocardium produces the typical myocardial lesion, healing by fibrous tissue scar formation. The coronary arteries react to freezing injury in a manner similar to major arteries in other locations. After freezing, healing is associated with smooth muscle proliferation and intimal hyperplasia, narrowing the lumen. These changes slowly regress during several months, but function as a conduit of blood continues while healing takes progress. Extensive clinical use of cryoablation in the treatment of the tachyarrhythmias is the stimulus for these many experiments.

Nerves

The detailed excellent report on histological changes in nerves caused by freezing by Denny-Brown *et al.* in 1945 reviewed the early studies which focused on the effect of tissue chilling, as in immersion foot and frostbite [42]. Ischemia was considered the likely factor of injury in these early studies. It was noted that the perineurium and epineurium survived freezing, though exhibiting an intense inflammatory reaction, and served as a conduit for the regenerating axons and the ability of nerves to regenerate after freezing was demonstrated.

In later years, interest in cryosurgery stimulated several investigations which showed similar results, even though more severe freezing techniques were used [13,18,25,123,212]. These experiments demonstrate that nerves are sensitive to freezing injury. The reaction of the nerve depends upon the temperature. Temporary desensitization of nerves occurs in the 0°C to -5°C range. And this minor injury is reversible- when the nerve is warmed, function will quickly return and the nerve will look normal. Somewhat colder than -5°C , return of function may be delayed for several hours or days. Permanent desensitization is produced at about -15 to -20°C and function is lost. If the nerve sheath is not disrupted, the function of sensory and motor nerves is very likely to return after a long time, perhaps months in the case of major nerves like the facial or the sciatic nerves.

Bone

Bone cells are very sensitive to freezing. Experiments with freezing the diaphysis of the canine femur by flowing liquid nitrogen through latex rubber coiled around the shaft, showed that the osteocytes die at temperatures colder than -5°C , and that the devitalized bone was slowly replaced by new bone over a period of about a year [64]. The repair depended upon the restoration of blood supply. As new blood vessels penetrated the devitalized femoral shaft, the dead bone was slowly replaced by new bone. During this process the form and function of the femur was preserved. However the strength of the bone was weakened by the process of resorption, so fractures were common in this weight-bearing bone about three months after

freezing. Popken *et al* used miniature cryoprobe cooled by liquid nitrogen to produce freezing in the tibia and femur of sheep [162,163]. The probe temperature was -180°C . At a distance of 1 cm. the temperature was -50°C . Necrosis was seen up to -10°C . In experiments in goats, Keijser *et al.* created gaps in the femoral diaphysis, froze the defect with a liquid nitrogen-cooled probe, and filled the gaps with bone grafts from the sternum [103]. The grafts resorbed in 10 weeks and did not accelerate healing of the cryosurgically treated bone. On the other hand, Pogrel *et al.* created defects in the mandible of pigs, froze the defect with a spray of liquid nitrogen, producing necrosis about 1mm deep, then filled the defect with autogenous bone from the chin [160]. The bone grafts enhanced healing.

Other experiments have shown that osteocytes are sensitive to freezing [110]. The process of repair of bone depends on revascularization, which features ingrowth of new blood vessels, resorption of dead bone, and replacement by new bone in a lengthy healing process, the time dependent upon the volume of bone frozen. The bone is weakened in the course of repair. The use of bone grafts or other supporting agents may add strength and facilitate the healing. These experiments have supported the present day treatment of selected bone tumors by curettage and adjunctive cryosurgery [131].

Brain

Local freezing of the brain has a long history. The initial experiments, beginning with the report of Hass and Taylor, were directed at the pathophysiology of disease and were reviewed in detail in several early reports [33,83,150,175,204]. The effects of freezing on the brain were shown in the detailed experiments reported by Walder, who used a liquid nitrogen-cooled probe, 2.6mm in diameter, cooled to -180°C , to produce freezing in the brain of cats [206]. Tissue temperatures were monitored by thermocouples placed about 1.5mm apart. Generally, a thermal equilibrium in the lesion was established in about 10 minutes of freezing. Thawing was followed by edema of the previously frozen tissue, but bleeding was infrequent. Vascular stasis and occlusion were observed. In the process of healing, the cellular infiltrate featured leucocytes and mesodermal cells. On the 5th day after freezing, a vigorous capillary network was developing and fibroblasts penetrated the lesion. Final healing required more than 2 weeks and often left a fluid-filled cyst. The reversibility of response at lesser freezing temperatures applied for less than 30 seconds was described.

Many of the investigators have produced local brain lesions by freezing for diverse purposes. Some experiments studied wound healing [85,195]. Other experiments focused on the therapy of brain injury or had the treatment of brain tumors in mind [10,31,32,118,217,219]. Progress in clinical application of cryosurgery to the treatment of brain tumors depends upon improved imaging control. Quigley *et al.* have shown that computerized tomographic-stereotactic techniques could be used to monitor brain lesions from freezing in dogs [164]. Recent studies by Tacke *et al.* have shown that, after freezing the frontal lobe in pigs, magnetic resonance (MR) imaging provided accurate monitoring of the freezing procedure and follow up of the subsequent necrosis [198]. In this study, MR imaging was considered superior to CT for monitoring cryoablation in the brain. Electrical impedance tomography may also prove useful [48].

In summary, freezing produced the typical circumscribed lesion in the brain. This provides a good model for pathophysiological studies of brain injury and has some potential for the application of cryosurgery to the treatment of brain tumors and other localized lesions if further improvements in imaging can be achieved.

The Eye

The eye received considerable attention in experiments in the 1960's. The use of freezing instruments on the lens in cats and rabbits prepared the way for the use of cryogenic techniques for the extraction of cataracts, which then stimulated other experiments. Lincoff and his associates applied freezing temperatures in the range of -10 to -20°C for a few seconds to the retina of rabbits, cats, and dogs [116]. This injury caused edema and congestion of the retina. The rods and cones and ganglion cell layers were destroyed, but the larger blood vessels and scleral fibers were resistant to freezing injury. Some cells of the pigment epithelium were destroyed. Healing was favorable. When used for this purpose, the testing indicated that the appropriate temperature of the probe was -20°C applied for 2 to 5 seconds. Geyer *et al.* reported that intraocular pressure will rise during cryotherapy in the eyes of animals [71]. Whitmore *et al.* reported that transcleral cryotherapy of the anterior retina in young rabbits slowed the growth of the anterior and posterior segments of rabbits eyes [211]. Later Axel-Siegel *et al.* reported that cryotherapy of the peripheral retina in rabbits resulted in elongation of the eye [5].

The early experiments established the present day usage of cryosurgery in diseases of the eye, including cataract removal, retinal detachment, and glaucoma, but applicability to the treatment of tumors remains limited.

The Uterus

Cervicitis and dysplastic cervical disease has been treated by cryosurgery for many years, so recent animal experimentation is not common. However, freezing uterine tissue causes the typical cryogenic lesion including the endometrium in the rabbit, as shown by Cahan and others [21,182]. Droegemueller *et al.* investigated the freezing of the Fallopian tubes as a potential method of sterilization [44]. In clinical practice, in competition with other ablative techniques, cryosurgery is used for uterine myomas and endometrial disease.

The Urinary Bladder

The effects of freezing on the urinary bladder were investigated in dogs by McDonald *et al.* in 1950, using pressurized carbon dioxide gas to cool a freezing plate, producing typical necrotic lesions [128]. Even at that time, his report included clinical use for bladder tumors. Recently using modern technology, Permpongkosol *et al.* investigated the morphological changes of urinary bladder cryoablation in pigs and produced a controllable transmural necrosis with single and repeated cycles [157]. No bladder perforation was produced. Though freezing bladder tumors had trials in a few patients years ago, recent clinical use is uncommon [124].

Other Tissues

Pomoski *et al.* used nitrous oxide-cooled apparatus to freeze the thyroid gland in rats [161]. In two weeks, necrosis and granulomatous inflammation were still present, but in four weeks after freezing, only lymphocytic infiltration and fibrosis were observed in the atrophic gland. The adrenal gland has been frozen in dogs and show the typical changes [183]. Cryosurgery of tubular structures, such as the common bile duct and the ureter, will cause a stricture in healing [7,63]. The vocal cords, after freezing, heal with fibrous scarring. Teeth respond to freezing in the same manner as bone, the viability of the dental pulp is restored by ingrowth of connective tissue through the apical foramens [51,76,84,112]. Salivary glands and tonsils heal with fibrous tissue replacement [23,142]. Little or no clinical use exists for cryotherapy of these tissues.

Immunologic Response

The immunological response to freezing was the subject of a recent comprehensive review by Sabel [178]. Therefore only a brief comment will be made. Shulman, Ablin and their associates

showed that tissue specific antibodies appeared in the serum of rabbits after freezing the prostate [1,189]. It was postulated that the rupture of cell membranes from freezing would release sufficient antigen to induce antibody formation. Interest in the immunological response increased sharply in 1970 when Soanes *et al.* described the remission of metastatic lesions following repeated freezing of prostate cancer in patients [193]. In the subsequent years, many experiments were directed at the nature and effect of the immunological response. Some investigators described benefit, but others did not, so merit has proven difficult to judge. Many investigators have found benefit from specific action on the immune system from stimulating the production of antitumor antibodies, which are cytotoxic, or by activation of cytotoxic T-cells or attributed benefit to cytokine response. Some investigators have thought that the immunological response could be enhanced by adjunctive agents.

Benefit also was demonstrated in experimental tumors after freezing by rechallenge by the tumor. Neel *et al.* showed enhanced tumor-specific immunity to experimental tumor produced by cryosurgery. In experiments with mice bearing tumors, challenge five weeks after primary tumor inoculation, those earlier treated by cryosurgery showed evidence of an immune response [148]. Neel and Ritts reported that the increase in tumor-specific immune response seen after freezing was augmented by excision of the tumor 24 hours after freezing. They suggested that most of the immunization that follows tumor necrosis produced by freezing occurs in the initial 24 hours [149]. Lubaroff *et al.* using a transplantable adenocarcinoma of the rat prostate, reported that freezing destroyed the tumor and that antibody and cellular immune responses were produced against the antigen of the tumor cells [119]. Rats with tumors treated by freezing and adjunctive BCG had sufficient immunity to protect them against rechallenge with the tumor. Miya *et al.* inoculated metastasizing rat mammary tumor into the thighs of female rats. Then seven days later, the tumor was subjected to freezing in two cycles by contact method at -170°C [137]. Resistance to tumor rechallenge induced by cryosurgery gradually increased after 6 weeks, suggesting anti-tumor immunity.

In contrast, many experiments have demonstrated no benefit which could be attributed to an immunological response [56,98,139,168,176,203,218]. Hoffmann and associates, using rats with a Dunning AT-1 prostate tumor, examining for immunologic benefit, found a measurable immune response by serum antibody levels, but no inhibition of tumor growth [86]. Their view was that the effect of cryosurgery in this tumor system was limited to the usual cellular and vascular changes. Some investigations showed enhanced growth after cryosurgery. Other adverse effects of an immunologic response have been reported. Blackwell, Chapman, and Washington and their associates, in a series of three reports on the effects of freezing about 35% of the liver in rats and sheep, described acute injury to the lung, thought to be due to cytokine release [16,27,208]. Seifert *et al.* freezing 50% of the liver in rats, noted increased serum cytokine levels, causing liver and kidney injury, likely mediated by cytokine released from the liver [184]. Joosten and associates also described elevated serum cytokine levels after freezing tumors implanted in mice [101].

In summary, these reports reflect a diversity of opinions about the benefits of the immunologic responses to cryosurgery. Though experiments with animal tumor demonstrate control of growth by freezing techniques, clinical experience has not shown benefit in tumors in humans from cryosurgery that could be attributed to an immunologic response. On the other hand, it is clear that the greater the volume of tissue destroyed by freezing, the greater the chance of postoperative lung and liver dysfunction and the greater the chance of a systematic response in the nature of multi-organ failure (cryoshock), which has been attributed to the release of cytokines and related products of tissue breakdown. Since an immunologic response of some type has been demonstrated in some animal experiments, further research in cryoimmunology is needed.

Adjunctive Therapy

Many recent experiments are directed at improving the efficacy of cryosurgery. The greatest opportunity for improved efficacy is in the management of the periphery of the cryogenic lesion where cell destruction is partial. Therapy adjunctive to cryosurgery, such as chemical agents, cytotoxic drugs, or irradiation should prove helpful in completing destruction of cells in the periphery of the frozen volume.

Cancer chemotherapeutic drugs are the most commonly used adjunctive therapeutic agents. Cancer chemotherapy has been used in patients who also have cryosurgical treatment for many years, that is, since the diverse clinical uses of cryosurgery for cancers in the late 1960's and 1970's. However, in those years, no effort was made to integrate the drugs into the cryosurgical therapy in a coordinated manner. The common clinical use of cytotoxic drugs with cryosurgery is via systemic administration and this has been generally beneficial, though not without risk when used in full therapeutic doses [174]. The mechanism of benefit has been investigated. Cooper and Fraser gave cyclophosphamide to mice with subcutaneously implanted sarcomas three days before a single freeze-thaw cycle of two minutes at a probe temperature of -70°C was applied to the tumor [37]. The combined therapy produced more cures than cryosurgery alone. They suggested that the use of cyclophosphamide potentiated cell-mediated immunity. A recent focus on the mechanism of beneficial action was provided by the *in vitro* experiments of Clarke *et al.* which showed that a combination of freezing and 5-fluoruracil produced a greater reduction in cell survival than either freezing or the drug alone, and this result suggested that the anti-cancer drugs promote apoptosis [30].

The experiments of Wang *et al.* studied the effect of cryosurgery and adjunctive 5-fluoruracil on apoptosis of glioma cells implanted in mice [207]. Though both cryosurgery and the drug can cause apoptosis independently, the combination enhanced the development of apoptosis in the glioma cells. Another mechanism of benefit was suggested by Mir and Rubinsky who, freezing melanoma cells *in vitro*, found that the cells became permeable to bleomycin [135]. This drug does ordinarily not permeate cells, so these experiments may have therapeutic implications. The benefit of adjunctive drug therapy was shown also in the interesting experiments of Forest *et al.* using a human lung cancer model in mice for treatment by cryosurgery, chemotherapy (Vinorelbine) or both [55]. The combined therapy had a better effect in reducing the tumor than either agent alone. A late cryo-induced angiogenesis was described.

Through the use of drugs as an adjunct to clinical cryosurgery is common and considered beneficial, the optimal dose and timing of delivery is not well defined. The key to effective drug delivery is in the thought that if drugs are administered during cryosurgery, the therapeutic agents will become sequestered or trapped in the tumor as microcirculatory failure develops. This timing of drug administration is supported by the experiments of Benson who, producing a cryolesion in the dog's tongue, noted that infused methylene blue solution became trapped in the previously frozen volume as the circulation failed in the thawing period [14]. He then extended this principle to the use of methotrexate as an adjunct to cryosurgery of oral cancer. Ikekawa and associates investigated the effect of combining cryosurgery and cytotoxic drugs in an experimental melanoma tumor in mice [93]. The anticancer drugs, peplomycin and adriamycin, were given intraperitoneally. When peplomycin was given after cryosurgery, the drug was trapped in the tumor. When the drug was given before cryosurgery, the drug was not trapped. Adriamycin was not trapped in the same manner. The results indicated that timing of the drug delivery was important. Homasson *et al.* have shown that bleomycin, given intravenously to patients with lung cancer is still trapped in the tumor 15 days after cryosurgery [91].

Locally administered chemotherapy, that is, placing the drug directly into the tumor, is an attractive therapeutic option in development. LePivert and his associates, using prostate tumor cells implanted subcutaneously in mice, after freezing the tumor in a single cycle, implanted microcapsules of 5-fluorouracil at the periphery of the tumors [114]. They described a dramatic inhibition of tumor growth.

Goel *et al.* using prostate cancer cells implanted in a dorsal skin fold and in the hind limb of mice as the test model, pretreated the tumor with soluble TNF-alpha topically or intravenously then froze in the tumor [74]. The result was an increase in the temperature threshold for necrosis and reduction in the size of the tumor by the combined therapy. In related investigation, freezing skin and tumor in a nude mouse model, Jiang *et al.* used TNF-alpha as an adjunctive agent and demonstrated that the use of TNF-alpha enhanced cell death by activation of both apoptotic and inflammatory pathways [99].

Radiotherapy is commonly used in the treatment of many tumors treated also by cryosurgery. However, as an adjunctive agent, the timing of delivery of irradiation is not clear. Experiments *in vitro* have shown that the radiosensitivity of cooled cells is increased [20]. One would expect that radiotherapy and cryosurgery, synchronized in some manner, would add to the efficacy of both.

The use of antifreeze proteins, which are glycoproteins that can modify the formation of ice crystals, have been investigated as an adjunctive agent. Using prostate cancer cells *in vitro*, Koushafar and Rubinsky have shown that the destructive effects of freezing are enhanced by antifreeze proteins [109]. The intracellular ice crystals, which form at warm freezing temperatures, cause mechanical damage to the cells. Experiments *in vivo* by Pham *et al.* using nude mice with subcutaneous metastatic prostate tumors, the preoperative injection of an antifreeze protein into the tumor before freezing enhanced the destructive effect of freezing [159]. Using flounder antifreeze proteins as an adjuvant to cryosurgery of subcutaneous tumor of rat prostate cells, Muldrew and his associates reported that a double freeze-thaw cycle with antifreeze protein injected with tumor provided a better tissue ablation than the double-freeze thaw cycle alone [138]. They suggested that the antifreeze protein might best be used by injection in the interval between freeze-thaw cycles as vascular stasis is developing, which would keep the agent in contact with the tumor for a longer time.

In short, the use of adjunctive agents is an attractive method of increasing the efficacy of cryosurgery. The choice of cytotoxic agent must be appropriate to the tumor in need of treatment. The effect is needed at the periphery of the tumor where freezing temperatures is not sufficient to kill cells and where manipulation of apoptosis is required. The timing of drug delivery for maximal benefit is still not clear. Perhaps continuous low dose chemotherapy is the best option.

SUMMARY

Cryosurgery had its beginning in the mid 1850's when irrigation with freezing solutions were used to treat advanced incurable cancer. Progress thereafter was slow in the first half of the 1900's when liquid air and carbon dioxide were used for freezing tissue in the first half of the 1900's. Modern cryosurgery began with the development of automated apparatus cooled by liquid nitrogen early in the 1960's. At that time, the major source of knowledge pertinent to cryosurgery was the prior and ongoing research on cryobiology in relation to frostbite and the preservation of cells and tissue. The mechanism of injury was known to be direct cell damage during freezing and the vascular stasis that develops following thawing. On this basis, the facets of cryosurgical technique quickly became fast cooling of tissue, slow thawing, and repetition of the freeze-thaw cycle. After extensive clinical trials in diverse tissues and organs and after a measure of enthusiasm for its results, cryosurgery fell into disuse for visceral tumors early

in the 1980's, but enjoyed a renaissance in the 1990's with the development of new apparatus and demonstration of the practicality of monitoring freezing with imaging techniques. In general, cryosurgical techniques have been judged to be safe and effective, though the incidence of recurrent disease in tumor therapy has compelled the research needed to improve efficacy [61].

In the modern era, experiments *in vivo* have defined some mechanisms of injury, the nature of the cryosurgical lesion, and the characteristics of wound healing, including differences in cell sensitivity to freezing. Experiments *in vitro* and *in vivo* have contributed to the understanding of the mechanisms of injury, including the importance of apoptosis as a cause of cell death and description of the use of diverse agents to modify the apoptotic response for therapeutic purposes. Advances in imaging have been noteworthy and are still evolving. With the use of multiple probes in the treatment of tumors in some organs, such as the prostate, computerized planning of the process of freezing is becoming desirable and perhaps necessary. Adjunctive agents of diverse types are being used in tumor therapy to enhance cell death in the penumbra of the cryosurgical lesion. Such agents may prove useful in selective cryosurgery in which the goal is to affect some cells but not others, as in the need to inhibit smooth muscle proliferation in vascular cryoplasty or in the stimulation of angiogenesis. In general, research in cryosurgery is progressing on a wide front, including molecular mechanisms of cell response, diverse aspects of technology, adjunctive therapy, and expanded applications to diverse tissues and organs.

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Table 1

LETHAL TEMPERATURES FOR CELLS

EXPERIMENTS IN-VIVO SINGLE FREEZE-THAW CYCLES				
REFERENCE		CELL/TISSUE	LETHAL TEMP	F-T CYCLE
First Author	REF Number			
	Year			
Gage	[66]	Osteocytes, bone, dog	-2°C	5 min F - slow T
Calams	[23]	Prostate, dog	-4°C	4 min F - slow T
Grady	[79]	Tumor, liver, rat	-4°C	Fast F - slow T
Gage	[70]	Melanocytes, skin, dog	-4°C	4 min F - slow T
Smith	[195]	Liver, rat	-15°C	3 min F - fast T
Rivoire	[176]	Liver, pig	-15°C	5 min F - slow T
LeFebvre	[115]	Cheek pouch, hamster	-18°C	1 min F - fast T
Dow	[45]	Prostate, dog	-20°C	Sec. F - slow T
Gage	[64]	Skin, dog	-40°C	3 min F - slow T
Yamada	[216]	Skin, mouse	-40°C	1 min F - slow T
Gage	[59]	Palate, dog	-40°C	3 min F - slow T
Neel	[150]	Sarcoma, mouse	-60°C	6 min F - slow T
Staren	[198]	Adenocarcinoma, rat	-40°C	15 min F - slow T
Chosy	[29]	Prostate, dog	-40°C	4 min F - slow T
Seifert	[189]	Cancer, mouse	-60°C	F-T
El-Shakhs	[53]	Liver, tumor, rat	-38°C	5 min F - slow T
Rupp	[182]	Kidney, pig	-15°C	15 min F - slow T
Seifert	[188]	Liver, pig	-15°C	20 min F - slow T

Table 2**EXPERIMENTS IN VIVO – REPEATED FREEZE THAW CYCLES**

REFERENCE			CELL/TISSUE	EFFECT
<i>First Author</i>	<i>REF Number</i>	<i>Year</i>		
Gill	[74]	1968	Rat Liver	Greater volume frozen
Myers	[143]	1969	Tumors, mice	No recurrence
Neel	[150]	1971	Sarcoma, mice	−60°C required
Whittaker	[214]	1975	Oral mucosa, hamster	Larger intracellular ice crystals
Gage	[59]	1978	Palate, dog	Increased destruction
Burge	[18]	1984	Cartilage, pig ear	Greater destruction
Rand	[172]	1985	Mammary cancer, mice	−50°C required
Ravikumar	[174]	1991	Tumor, liver, rat	−35° required
Dilley	[43]	1993	Liver, sheep	Moved necrosis close to border
Pogrel	[165]	1996	Skin, rat	Wider destruction; −20°C lethal
Staren	[198]	1997	Breast cancer, rat	Enhanced destruction
Wooley	[215]	2002	Kidney, dog	Enhanced destruction
Seifert	[188]	2003	Liver, dog	Larger lesion
Kollmar	[108]	2004	Liver, pig	Enhanced destruction