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EDITORIAL

History, ethics, advantages and limitations of experimental models for hepatic ablation

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Abstract

Numerous techniques developed in medicine require careful evaluation to determine their indications, limitations and potential side effects prior to their clinical use. At present this generally involves the use of animal models which is undesirable from an ethical standpoint, requires complex and time-consuming authorization, and is very expensive. This process is exemplified in the development of hepatic ablation techniques, starting experiments on explanted livers and progressing to safety and efficacy studies in living animals prior to clinical studies. The two main approaches used are *ex vivo* isolated non-perfused liver models and *in vivo* animal models. *Ex vivo* non perfused models are less expensive, easier to obtain but not suitable to study the heat sink effect or experiments requiring several hours. *In vivo* animal models closely resemble clinical subjects but often are expensive and have small sample sizes due to ethical guidelines. Isolated perfused *ex vivo* liver models have been used to study drug toxicity, liver failure, organ transplantation and hepatic ablation and combine advantages of both previous models.

Key words: Liver; Ablation; Experiment; *Ex vivo*; *In vivo*; Perfusion

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INTRODUCTION

Progress in science and medicine depends on new ideas being developed, tested and introduced into mainstream practice. Animal experimentation is used not only in medicine and surgery but also in science, the pharmaceutical industry, the military and educational establishments. In medicine it is essential to establish beyond doubt the safety of a drug, instrument or procedure before it even begins phase 1 of clinical trials. To establish the safety of this heterogeneous range of treatments the medical profession has frequently had no option but to the use of live animals. Although this practice is highly undesirable and many people are morally opposed to these experiments, unless and until viable alternatives are available legislation prevents the introduction of these new drugs and procedures without this prior testing. Indeed virtually all drugs and surgical treatments that are in use today have been tested on animals.

Although the best treatment for liver tumour remains surgical resection[1] the majority of patients are still affected by unresectable lesions. To these patients hepatic ablation provides an opportunity to increase survival[2]. Experimental research over the last twenty years has seen the development of a number of ablative modalities and

several of these have been applied clinically[3,4]. Ablation experiments are now focused to increase the diameter of the ablation zone to achieve better tumor margins[5-12], or to conduct safe ablations on lesions positioned close to major vessels[13-16]. Numerous experimental models are available, each with different advantages, disadvantages and ethical implications. It is therefore imperative that the researcher that wants to explore the field of experimental hepatic ablation is aware of their characteristics.

HISTORICAL BACKGROUND OF ANIMAL EXPERIMENTATION

The Greeks almost 2500 years ago described the earliest recorded animal experiments. Aristotle (384-322 BC), and Erasistratus (304-258 BC)[17,18] both performed studies on live animals and in 2nd century Rome Galen who is known as the father of vivisection dissected live goats and pigs[19]. These studies continued into the Roman era and were then passed on to medical schools in Arabia. The practice died out after this and was absent completely in the Dark Ages only being revived in Italy in the 16th century. Subsequently live animals have been used throughout history to study a wide range of problems and to assess new treatments particularly drugs and vaccines. In the 17th century, many pivotal discoveries came to light because of these studies including the understanding of lung function and the circulation of the blood. In the 1840's general anesthesia emerged (initially ether and chloroform) and it became possible to study unconscious animals. In 1881, 250 experiments were carried out in Britain, the first year that records were kept of the procedures carried out on animals. The following year (1822) the first animal protection law was passed in Britain and in 1876 the Cruelty to Animals Act came into being and was the first law specifically designed to regulate animal testing.

Many advances in our understanding did result from these animal studies including those in basic science and also medicine. These included Antoine Lavoisier demonstrating that respiration was a form of combustion using guinea pigs in calorimeters[20], Stephen Hales measuring blood pressure in the horses, and in the 1880's Louis Pasteur demonstrating the concept of "germs" by giving anthrax to sheep[21]. Also in the 1880's and 1890's Emil von Behring was able to isolate the diphtheria toxin and not only demonstrate its effects in guinea pigs but also, by 1898 produce immunity by the injection of a mixture of toxin and antitoxin, for which he was awarded the Nobel Prize in Physiology and Medicine in 1901. In 1921 Banting ligated the pancreatic duct of dogs and demonstrated that pancreatic isolates could be used to keep these diabetic animals alive, and by 1922 working with John Macloed he isolated insulin from bovine sources and famously treated Leonard Thompson, a 14-year-old diabetic boy with diabetes[22].

OBJECTIONS TO LIVE ANIMAL EXPERIMENTS

Despite these advances and those that took place subsequently many people remained understandably opposed to any form of live animal experimentation. Claude Bernard was known as the "prince of vivisectors" and the father of physiology[23] but ironically his wife Marie Francoise Martin founded the first anti-vivisection society in France in 1883 and famously wrote in 1865 that "the science of life is a superb and dazzlingly lighted hall which may be reached only by passing through a long and ghastly kitchen[23,24]. Opposition to animal experimentation continued and many eminent scientists voiced their disapproval. Charles Darwin in 1871 wrote to Ray Lancaster in reply and stated "You ask about my opinion on vivisection. I quite agree that it is justifiable for real investigations on physiology; but not for mere damnable and detestable curiosity. It is a subject which makes me sick with horror, so I will not say another word about it, else I shall not sleep tonight"[25].

Objections to live animal experiments emerged in the United States in the 1860's and Henry Bergh founded the American Society for the Prevention of Cruelty to Animals. The first movement which specifically opposed vivisection was the American Antivivisection Society, which was founded in 1883. The continued opposition to the use of animals together with the dramatic increase in the number of procedure that were being performed (in 2002 in the United Kingdom 2.7 million live animal experiments were authorized and in the United States, it is estimated that between 19 and 29 million experiments were performed although exact estimates were difficult because 90% of the animals were rats, mice and other species recently exempt from legislation) encouraged many researchers in science and medicine to seek other methods to test drugs and procedures and much of this ethos has been incorporated into legislation or guidelines. Although British law requires that any new drug be tested on at least two different species of animals (one must be a large non-rodent), United Kingdom regulations in respect of animal experimentation are very strict and the Animals Act of 1986 requires that no animal experiments be conducted if there is a realistic alternative. It also exhorts investigators to examine their research carefully to determine the smallest number of animals that may be used to answer the questions posed by the research.

There remains however a dilemma with both ethical and legislational dimensions. All scientists agree that animal experimentation is unacceptable if there is a viable alternative but legislation will not allow the introduction of new products without rigorous testing. In 1988, the American Medical Association published a white paper defending biomedical research in animals[26], and stated that "In fact, virtually every advance in medical science

in the 20th century, from antibiotics and vaccines to antidepressant drugs and organ transplants, has been achieved either directly or indirectly through the use of animals in laboratory experiments". In Europe European Centre for the Validation of Alternative Methods was established in October 1991 by a communication from the Commission to the Council and the Parliament. The aim was to try and organize research and exchange information to limit the need for animal experimentation. As defined in the communication it was felt that this could be achieved in four ways: (1) to coordinate the validation of alternative test methods at the European Union level; (2) to act as a focal point for the exchange of information on the development of alternative test methods; (3) to set up, maintain and manage a database on alternative procedures; and (4) to promote dialogue between legislators, industries, biomedical scientists, consumer organizations and animal welfare groups, with a view to the development, validation and international recognition of alternative test methods.

Although there has been considerable progress in these areas, notably with the use of fresh and cryo-preserved cells (particularly hepatocytes) problems remain when it is necessary to investigate a treatment prior to its use inpatients. The problem is particularly acute in the field of biotechnology in which many of the devices that are proposed and developed are potentially harmful and effects in living tissue are essentially unknown. Today it is certainly possible to computer model some of the effects (widely used in the pharmaceutical industry to predict the usefulness and safety of new drugs) but many results remain unpredictable. Fresh tissue can be used and will provide answers if the procedure takes a short time and its effects and results do not rely on changes (particularly at the cellular level) that require interaction with living cells or evolve over a significant time period (several hours or longer). Unfortunately many devices do produce changes due to interaction with normally vascularized tissue and frequently take prolonged periods to produce effects. With these the only way to date that the results could be examined in detail was in living animals. In addition although prototypes could be studied in small animals instruments that have been developed for human use because of their size can often only be studied in large animals.

LIVER ABLATION EXPERIMENTAL MODELS

The number of patients with liver lesions, which are potentially suitable for treatment, continues to rise with advances in critical care, anesthesia, surgery and oncology. At present, the gold standard remains surgical resection that produces long-term survivors and cure in a significant number of patients. The advent of laparoscopic resection has further widened the indications but by far the most attractive concept remains ablation of the liver (for primary or secondary tumors) containing the tumour. The ablation techniques currently in place for the treatment of liver tumors include radiofrequency ablation (RFA), microwave ablation (MWA), cryotherapy, percutaneous ethanol injection, high intensity focused ultrasound, interstitial laser photocoagulation, electrolysis and bimodal electric tissue ablation[4]. The additional availability of methods for real-time monitoring to ensure that a sufficient volume is ablated (to engulf the tumor and reduce the likelihood of local recurrence) has seen an upsurge in the number of procedures performed and the consequent emergence of survival data that are very encouraging. In addition there is evidence that ablative techniques produce immunologically active components that may confer some extra survival advantage but also systemic effects with potentially harmful consequences.

The development of any new hepatic ablative therapy is a long process that requires a detailed understanding of the basic physical and chemical properties of the ablative modality and their application in clinical settings. It includes designing equipment suitable for clinical application, identifying methods of assessing tissue response to ablation, optimizing treatment monitoring, exploring local and systemic effect of the ablation, and finally, demonstrating the safety and efficacy in humans. Understanding of the tissue temperature changes and radiological evolution of lesion generated by each ablative modality is crucial before it can be used in a clinical setting. Further problems occur with some of the techniques that rely on temperature changes for their effectiveness. Particularly with RFA and MWA the effect can be significantly attenuated in the vicinity of large blood vessels due to the "heat sink effect" where the large flow in the vessels conducts heat away sufficiently rapidly to interfere with the treatment and potentially leave viable tumor cells around these vessels. Flows within the hepatic vessels have been shown to reduce the ablative energy via heat sink effects resulting in a greater energy requirement when ablating in close proximity to larger vessels with an associated higher risk of vascular and bile duct complications[15,27]. For the same reasons, thermal ablation techniques particularly are influenced by the effects of the separate occlusion of the hepatic artery, portal vein or both (Pringle maneuver). This delicate balance between safety and efficacy of ablation has been the focus of many experimental studies[15,28,29].

One further attraction of ablative techniques is the fact that they can often be performed percutaneously or laparoscopically and considerable effort has been expended in the development of suitable probes. These probes can be complex and are generally modeled initially on computers to predict the likely outcome after the delivery of a set amount of energy. Unfortunately the alterations in tissue resistance and electrical and thermal conductivity consequent upon the use of the probes, together with the complex designs (and frequent use of multiple probes or probes that assume unpredictable

shapes after deployment) means that to date it has been essential to test the devices in animal models. These will also be necessary to "calibrate" all new devices and produce standard dose-response curves, so that in addition to real-time monitoring a prediction can be made about the amount of energy that needs to be delivered to ablate a predetermined volume.

The increasing popularity and deployment of these techniques means that to accomplish these aims would require the use of a large number of experimental animals, and because of this it is valuable to consider whether there is an alternative approach that can produce reliable data of sufficient quality to facilitate the incorporation of these methods into clinical practice without the need for subsequent large animal studies. *Ex vivo* perfused liver models have been developed recently for the study of the hepatic toxicity of new pharmacological agents[30-32], and in an attempt to improve the outcome from organ transplantation. Organ transplantation models, which use pulsatile perfusion after harvesting, would seem to offer an excellent alternative that could closely mimic the *in vivo* situation and allow data to be collected for up to 24 h. In a sufficiently well developed perfusion model in which parameters can be accurately controlled it seems likely that data generated would be genuinely useful and achieve the aim of avoiding the use of live animal models.

EX VIVO NON-PERFUSED MODELS

The most commonly used model for the study of hepatic ablation is liver explanted from animals. Bovine and porcine livers are most frequently used because of their similarity to human livers in terms of size and density, and because they are farm animals that are readily available and inexpensive. In addition long-term use has resulted in a familiarity with the animals' anatomy that is essential in studies on the liver where the lobulation and blood supply are an important determinants of outcome. Although the organ is not perfused the position of the ablation in respect of vessels and bile ducts remains important. Their size also allows a number of ablations to be carried out on each individual model, which minimizes the number of animals required.

These models can be used to examine the relationship between ablative energy and lesion size, to assess the effect of different antenna configurations on lesion size and to study the dielectric properties of liver tissues[33-36]. Crocetti *et al*[37] used *ex vivo* calf livers to assess the feasibility and validity of fusion imaging that combines ultrasound and computed tomography in the monitoring of RFA. Unfortunately the results of this study were not directly transferable to the clinical setting because of the absence of respiratory excursion and subject motions of this model. The absence of perfusion within *ex vivo* models also limits their ability to assess the effect of blood flow on the size of the lesion created by ablation with a set amount of energy. Furthermore detailed studies of the cellular changes in the ablated area cannot be conducted due to the ischemia produced by explantation and the rapid cessation of metabolic and physiochemical processes.

IN VIVO MODELS

In view of the limitation of *ex vivo* models particularly with respect to the absence of blood flow, *in vivo* animal models have been widely used for the study of hepatic ablation. Data generated from these studies are very reliable and the close similarity to results in humans allows the experience to be directly transferred to clinical practice. Small laboratory animal models such as murine models are easier to handle but have significant differences in organ size, function and anatomy compared to the human liver. The relatively small organ size also limits the number of ablations that can be carried out per liver. In contrast, *in vivo* porcine liver closely resembles human livers in size, vascularity and metabolic function and although the shape is different the organ is also lobulated in a similar way to the human liver. This allows comparisons to be drawn with respect of the positioning of probes, real dosage levels and consequences of treatment. The result is that the porcine model is by far the most commonly used at present for the final evaluation of all ablative techniques.

The two major approaches to date for the study of hepatic ablation in animals, *ex vivo* non-perfused liver models and *in vivo* animal studies, and their respective advantages and limitations are compared in Table 1. A number of studies have been carried out simultaneously on *in vivo* and *ex vivo* non-perfused models to try and assess the magnitude of the changes produced and also to evaluate the specific differences. The volume of the ablated regions is always significantly smaller in the *in vivo* models when similar energy of ablation is applied[5,38,39]. These findings can probably be attributed to the presence of vascular flow, which removes some of the energy (heat) during the treatment in the *in vivo* models and hence these models are used to examine the influence of vascular occlusion on the size of ablated area. *In vivo* models also allow histological study of different zones, and the evolution of these zones in lesions generated by thermal ablations.

Results generated from *in vivo* models are directly transferable to clinical practice and are at present the gold standard for pre-clinical experimental studies of hepatic ablation. However the use of living animals requires specific laboratory facilities and research teams with expertise in anesthetizing and handling different species. The size and temperament of larger animal models such as porcine and bovine models poses practical problems when animals need to be anesthetized, moved or examined post-procedure (particularly if blood sampling is required).

Table 1 Advantages and limitations of *ex vivo* and *in vivo* models for experimental study of hepatic ablation

Models	Applications	Advantages	Limitations
Ex vivo (non perfused) models	Compare the efficacy of different antenna configurations Trial of different energy setting	Allow histological examination of whole lesion to study zones of ablation Cheap Larger study sample size Easy to manipulate during experiment Does not require ethical approval/animal license	Non-physiological Homogenous parenchyma Absence of respiratory excursion and subject motions Lack of cooling effect secondary to tissue perfusion Unable to study heat sink effect
In vivo models	Study of lesion evolution over time Histological examination of lesion Study of heat sink effect and the effect of bile duct cooling Study of systemic responses to ablation Study of the effect of large volume ablation (in larger animals)	Small animals Easier to handle Cheaper Ability to have larger sample size	Small animals Small volume of liver Limit number of ablation on each liver Not suitable for the study of large volume ablation
		Large animals Closer resemblance to human liver in terms of size and physiology More ablations can be carried out in each liver	Large animals Size and temperament poses challenges during anesthesia Difficult vascular access in porcine models Also limited by strict ethic regulation Small study sample size
			Common limitations Expensive Expertise in animal handling and anesthesia is required Duration of study is limited to the lifespan of the model Absence of interacting organ systems which may have a role in generating systemic response Unable to assess the impact of ablation on end organs
Isolated perfused *ex vivo* liver models	Study of lesion evolution over time Study of heat sink effect and the effect of bile duct cooling Study of early inflammatory response	Cheaper than *in vivo* experiments Sophisticated and accurate manipulation of hepatic inflow (e.g., Pringle manoeuvre) Does not require ethical approval Greater control of perfusion characteristics (e.g., portal vein and hepatic arterial flows and pressures)	Perfusion circuit itself may activate some degree of systemic response

EX VIVO PERFUSED MODELS

Ex vivo non-perfused and *in vivo* models have significant advantages with respect to specific scientific questions but an alternative model, the isolated perfused *ex vivo* model, theoretically would have most of the advantages of both. A normothermic liver perfusion system using autologous blood maintains physiological and metabolic functions and the hemodynamic changes resemble those of *in vivo* models with good preservation of liver architecture[40-42]. The organ would be perfused over a number of hours, and within this period valuable data could be produced within a relatively inexpensive model with no ethical problems and no requirement for licensing (Table 1)[43-45]. This should permit the histological study of lesions generated by hepatic thermal ablation, real-time monitoring of the evolution of the lesions and experiments designed to study the "heat sink" effect with different modalities. In addition, with a sufficiently sophisticated and stable model it should also be possible (by venous sampling) to study the early inflammatory responses following hepatic ablation[44,46].

Liver perfusion studies date back as far as the 19th century when they were first performed for research on physiological function of the liver[47]. A number of studies have been conducted subsequently to establish valid models of liver perfusion using asanguineous perfusates or autologous blood[41,48-50]. These models have been used for physiological research, treatment of liver failure, studies of metabolism and toxicity of new pharmacological compounds and in transplant units for the recovery and preservation of organs, which are to be implanted[51-53]. Hildebrand *et al*[54] have advocated the use of a perfused *ex vivo* liver model for the training in laparoscopic RFA.

For the *ex vivo* perfused model the main limitation is the limited lifespan and the determination of intervals during this lifespan when valid data can be extracted. Nevertheless perfusion models, and particularly those using autologous blood and pulsatile perfusions, have become very sophisticated and physiological parameters can be very accurately maintained for many hours. The interaction of blood with non-biological surfaces of the perfusion circuit does activate a number of biological pathways producing some degree of systemic response and interpretation of responses to ablation will have to take these into account. However the response will be the same for each experiment and should not interfere with comparisons of different dose responses or different modalities[55]. The absence of other interacting

organs may also affect the extent of systemic responses following hepatic ablation but this model still potentially represents an exciting new method of studying these new devices while avoiding the need to use live animals. The true potential and weakness of an isolated perfused liver model for the study of hepatic ablation remain to be seen (and will be determined by future research) but recent advances have certainly developed the model to the stage that merits this research[56].

FUTURE DIRECTIONS AND ADVANCES

To overcome the limitations of disconnecting the liver from the remaining organs, Chung *et al*[56] recently created the first multiorgan *ex vivo* perfused model in which the liver was serially connected with the kidney. The viability of both organs and the inflammatory reaction elicited have been investigated in two separate studies[56,57]. The addition of the kidney to the circuit improved the biochemical milieu of the circuit and consented a more physiological environment for those experiments requiring strict conditions[57]. At the same time, the combination of two organs in the circuit did not increase the cytokine production compared to the classic "liver-alone" circuit[56] and therefore could be used to test the inflammatory reaction produced by ablative techniques[44] without significant interferences by the newly added organ.

CONCLUSION

The key advantages of isolated perfused liver models are the avoidance of live animal experiments, the ability to control accurately physiological parameters and to analyze morphological changes following hepatic ablation. In addition detailed study of the "heat sink" effect and changes consequent upon bile duct perfusion can be assessed. Other organs and tissues from the same animals from which the livers are procured can also be used in other research leading to an overall reduction in the number of animals required. Validation of the role of isolated perfused liver models for experimental studies of hepatic ablation is essential to establish their position in this field of research.

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