Review

The Influence of Extremely Low Frequency Magnetic Fields on Cytoprotection and Repair

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Ischemia-reperfusion injuries, such as those suffered from various types of cardiovascular disease, are major causes of death and disability. For relatively short periods of ischemia, much of the damage is potentially reversible and in fact, does not occur until the influx of oxygen during the reperfusion stage. Because of this, there is a window of opportunity to protect the ischemic tissue. Here, we review several mechanisms of protection, such as heat shock proteins, opioids, collateral blood flow, and nitric oxide induction, and the evidence indicating that magnetic fields may be used as a means of providing protection via each of these mechanisms. While there are few studies demonstrating direct protection with magnetic field therapies, there are a number of published reports indicating that electromagnetic fields may be able to influence some of the biochemical systems with protective applications. Bioelectromagnetics

Key words: electromagnetic; heat shock proteins; opioid; cardioprotection; ischemia-reperfusion; blood flow; noninvasive clinical applications

INTRODUCTION[1]

Heart disease and stroke are major illnesses in the developed world, with 8 in 10 Canadians having at least one risk factor for developing cardiovascular disease and cardiovascular disease underlying approximately one third of all deaths [Heart and Stroke Foundation of Canada, 2003]. In the future, these problems will be exacerbated by the aging population.

Up to two-thirds of cardiovascular deaths are related to ischemic injuries: the reduction or blockage of blood flow, which can have many underlying causes, such as narrowing of the blood vessels or blockage by a clot or other obstruction (embolism). The restoration of blood flow is of prime concern for treating cases of

[1]**GLOSSARY**

Cross-protection: Using different sources of cellular stress to induce the protection seen from preconditioning. For example, using a brief period of mild hyperthermia to induce protection against a subsequent, potentially lethal period of ischemia. The existence of this phenomenon indicates that certain stress response factors, such as HSPs, are not stress-specific.

HSP: Heat shock protein, a class of proteins that normally act to chaperone the proper folding of nascent proteins, but whose expression is also increased in times of cellular stress, such as hyperthermia. They can also repair or protect against some types of cellular damage.

HSC: Heat shock cognate, a type of heat shock protein that is constitutively expressed. Note that most HSPs are present at some level in the cell at all times, and even constitutively expressed isoforms have some degree of stress-inducibily.

Mu Metal™: A metal with a very high magnetic permeability, it is often used to shield stray magnetic fields.

Preconditioning: The process of using one mild stress to protect against a more serious one to come; also known as "stress hardening." The first mild stress can prompt the stress response, which can create protective/reparative factors, such as HSPs, which can prevent cell death from a second stress that may be potentially lethal.

SAR: Specific absorption rate, a measure of energy deposited in tissue by electromagnetic radiation.

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ischemia, and great advances have been made in ''clot busting'' treatments in recent years. While tissue in which blood flow is never restored will become permanently infarcted (die), tissue that is reperfused (restored blood flow) can still accrue a great deal of damage, as much of the damage in ischemia-reperfusion occurs after reperfusion. Protecting tissue prior to this reperfusion insult is thus an important research area.

Conventional treatments, particularly tissue plasminogen activator (tPA) for clot removal, are typically administered within 3 h of symptom onset [recommended door-to-needle time of 60 min; see Guidelines Advisory Committee guidelines, 2003]. Thus, some amount of time is spent between hospital admission and administration of a thrombolytic agent, giving a window of opportunity for protective therapies to be applied. [Guidelines Advisory Committee guidelines, 2003; Heart and Stroke Foundation of Canada, 2003].

Preconditioning studies have shown that if a mild reperfusion injury is suffered, endogenous defence mechanisms will be activated and can reduce damage suffered from a second, more serious ischemic insult [Pasupathy and Homer-Vanniasinkam, 2005]. These techniques can be useful in cases where potentially fatal injuries can be foreseen, such as prior to surgery or transplantation. Furthermore, the endogenous defences are robust enough to protect against a number of different types of stressors, so that one stress such as hyperthermia may induce protection against a different subsequent threat, such as a reperfusion injury, a phenomenon known as cross-protection. However, this technique is only useful when advance knowledge is available, and it involves damaging—though possibly only slightly—the very tissue one is trying to protect.

More direct ways of inducing these endogenous protective mechanisms are also being considered through pharmacologic and transgenic means. Unfortunately, any blood-borne pharmaceutical agent will not have a good chance of inducing protection because it will depend upon blood flow that is, by definition, reduced. Transgenic methods suffer from this drawback as well, except in the case where the vectors are present in the tissue prior to the injury or injected directly into the site of injury. This may not be socially acceptable, however, as currently there are significant public concerns regarding transgenic treatments.

Another alternative, magnetic field therapy, is the focus of this review. Some magnetic field exposures have been shown to effectively treat a number of other conditions, particularly for bone nonunions and wound healing [for review, see Shupak et al., 2003]. It has been suggested that magnetic fields, which can be applied early and remotely, that is, without relying on the blood flow to carry a substance to the site of injury, can protect or heal tissue in the midst of an ischemia-reperfusion injury. Several mechanisms for the potential effectiveness of such therapy have been proposed, including heat shock protein induction to protect cells from damage when the wash of free radicals occur, increased peripheral blood flow to reduce the amount of ischemia, and nitric oxide effects, which can improve blood flow and reduce inflammation.

ISCHEMIA-REPERFUSION INJURY

Ischemia, a lack of blood flow to tissue, is a generalized insult to tissue involving deficits in oxygen and nutrient delivery and the build-up of metabolic by-products. It is often caused by a blockage in a blood vessel by a blood clot or fatty plaque, particularly in ischemic heart disease or ischemic strokes, but can also occur in many other situations, such as during organ transplantation.

A number of therapies have been approved and are recommended for clinical use that attempt to minimize the length of time that tissue must spend in an ischemic state, such as thrombolytic agents (clot-busters) and vascular stenting/angioplasty [Ryan et al., 1999; Guidelines Advisory Committee guidelines, 2003; Kloner and Rezkalla, 2004]. Significant tissue damage can still occur, even when these interventions are undertaken within a short period of time, because much of the damage is suffered at the reperfusion stage.

During the ischemic phase, the cells continue to metabolize and quickly become hypoxic, though not necessarily absolutely free of oxygen (anoxic). Energy stores are depleted and certain active processes, such as the maintenance of ion concentration differences, shut down. This may be particularly important for the calcium balance, as internal calcium pumps such as those on the mitochondria or sacroplasmic reticulum may work against the increasing calcium concentration arriving from the extracellular space, speeding the depletion of energy in the cell [Maxwell and Lip, 1997].

In the mitochondria oxygen is reduced to water in the process of producing adenosine triphosphate (ATP), the major energy-carrying molecule in the cell. However, some of the oxygen follows a different pathway to form superoxide (O_2^-), which is dismutated to H_2O_2, which is further converted to harmless H_2O by several pathways. During ischemia, these antioxidant defences may become eroded and H_2O_2 may build up and convert to more reactive and harmful hydroxyl (OH^-) radicals [Becker, 2004]. The pathway that leads to the formation of superoxide radicals in the mitochondria may also be favored over the harmless H_2O one

under ischemic conditions [Maxwell and Lip, 1997; Li and Jackson, 2002], due to the influence of pH or calcium concentration.

As the cell consumes more of its energy reserves, ATP is broken down into ADP, AMP, and finally adenosine, which is converted to xanthine [Maxwell and Lip, 1997]. The xanthine dehydrogenase/oxidase enzyme will break down xanthine and in the process alter itself so that it will create superoxide radicals in the presence of oxygen, particularly during ischemic conditions. So, upon reperfusion, the enzyme can react with oxygen and release a burst of radicals [Li and Jackson, 2002]. However, this particular mechanism may not apply to human myocardium, although it is present in other cell types [Maxwell and Lip, 1997].

If blood flow is restored, the influx of oxygen almost immediately reacts with these built up metabolic byproducts and compromised electron transfer chains, and a surge of superoxide anions and other free radicals is produced. Iron may play a role in this process, facilitating the conversion of superoxide to the more harmful hydroxyl radical. [Kruszewski, 2004] This is a very rapid process, and studies [see Becker, 2004 for review] indicate that the free radical concentration peaks within about 20 s of reperfusion. This burst in free oxygen radicals corresponds to the finding that most of the loss in cellular viability and lipid damage [Maxwell and Lip, 1997; Li and Jackson, 2002] occurs after reperfusion, which indicates that some of the damage suffered from ischemia-reperfusion is preventable.

While the free radical mechanism of ischemia-reperfusion injury is widely accepted and useful for illustrating the nature of some treatments, it is not the only mechanism whereby tissue can accrue damage from ischemia-reperfusion. Neutrophil responses, calcium imbalances, and for the heart in particular, arrhythmias and myocardial stunning are other mechanisms that are beyond the scope of this paper. Note that these mechanisms are not necessarily independent of the free oxygen or free nitrogen radical mechanisms, for example, calcium imbalances may exacerbate superoxide production by the mitochondria after reperfusion, and free oxygen radicals may damage calcium channels [Maxwell and Lip, 1997].

MAGNETIC FIELD THERAPY

Some studies have suggested that magnetic fields can prevent or repair damage suffered following an ischemia reperfusion injury. Albertini et al. [1999] found that applying a 3 mT, 75 Hz pulsed extremely low frequency (ELF) magnetic field reduced the amount of permanently damaged myocardium (infarct size) following a permanent ligation of the left anterior descending coronary artery in rats. However, this effect was only seen in the short term (18 h), and no significant difference in infarct size was seen between control and exposed animals after 6 days. This result may have been expected, as the ligation was permanent. Magnetic fields may delay cell death from ischemia, or improve collateral flow to reduce the size of the ischemic penumbra, but certainly cannot preserve tissue for 6 days without blood flow, although angiogenesis may have occurred [Yen-Patton et al., 1988]. The authors also tested ischemia reperfusion injuries; however the pulsed magnetic fields were not applied until after surgery, so there was no opportunity for protection to be conferred prior to the reperfusion.

Protection during this window has been demonstrated by Grant et al. [1994] in a rabbit model of cerebral ischemia. During a 2 h occlusion of the left internal carotid, proximal left anterior cerebral, and proximal left middle cerebral arteries, a pulsed 75 Hz sawtooth electromagnetic field was applied to the rabbits, similar to the one applied in the Albertini et al. study above. This pulse was applied prior to reperfusion, however, and a significant decrease in damaged neural tissue resulted.

A similar set of studies by DiCarlo et al. [1998, 1999a, 2000a,b,c; DiCarlo and Litovitz, 1999] has also found a protective effect of magnetic fields on chick embryos. In a series of experiments involving extremely low frequency (ELF) magnetic fields, they found that the survival rate of chick embryos was increased after being exposed to a potentially lethal hypoxic situation—sealed inside a plastic bag—until the survival of the control embryos dropped below 50% (after reoxygenation, control survival decreased further to 24% while exposed survival was approximately 45% [DiCarlo et al., 2002]). ELF magnetic fields (60 Hz, 8 μT) were applied an hour prior to the hypoxia. Looking at the parameters of the exposure as well as other effects in an attempt to shed light on the potential mechanism, they found that the magnetic field preconditioning could also protect against ultraviolet light damage [DiCarlo et al. 1999b,c, 2002], which may indicate a free oxygen radical scavenger mechanism. These findings were successfully reproduced by Björåsen et al. [2004] at 50 Hz (10, 50, or 100 μT rms), including the finding that vertical fields were more effective at inducing protection than horizontal ones (the long axis of the egg being vertical, cf. DiCarlo et al., 2000a).

In a longer term study on broad band radio-frequency fields (0.2–200 MHz) at two different magnetic field intensities (11.4 and 36.1 μT) given to rats for short exposures (<10 min) daily over 3 weeks, Ronchi et al. [2004] found changes in isolated heart function following ischemia-reperfusion in the lower

field intensity exposure. These functional changes indicated that the magnetic field treatments decreased ischemic tolerance relative to controls. Paradoxically, the low field (but not the high field) condition increased Hsp70 levels in the myocardium; this protein is generally considered to have protective effects. However, this study used a low-flow ischemic condition, rather than a more severe blockage of blood flow. The ischemic insult did not produce any appreciable apoptosis in any of the conditions, and necrosis/infarct was not examined. Thus, while this magnetic field exposure appears to have deleterious short-term effects on functional parameters, effects on long-term survivability/cell death, especially due to more severe ischemia, is not clear. Also, DiCarlo et al. [2002] found that chronic exposures decreased protection, although the length of the daily exposures was much longer than in this study.

While the number of published studies on the ability of magnetic field therapy to reduce ischemia-reperfusion injuries is limited, there are reports of effects on various biochemical systems that will be discussed below. With our knowledge of the mechanisms behind reperfusion injury and the effectiveness of more conventional treatments targeting these systems, we may consider some of these studies as proxies for the overall influence of magnetic fields, at least as far as considering the area for further study.

MECHANISMS: HEAT SHOCK PROTEINS

Heat shock proteins (HSPs) are a class of proteins that are widely known to protect other proteins and the cytoskeleton from damage through a variety of mechanisms. There are several families of HSPs, identified by their molecular mass, each family acting with its own unique mechanism. For nomenclature purposes, capitalized *HSP* is used to refer to a gene or general reference to a class of proteins; mixed-case Hsp to refer to a specific protein; and lower-case italicized *hsp* to refer to nucleic acids. For example, the *HSP70* class contains the proteins Hsp70, Hsp72, and Hsp75; the HSP70 mRNA *hsp72*, etc.). For clarity, the explicit ''HSP70 protein'' and ''HSP70 mRNA'' have been used in many instances instead of ''Hsp70'' and ''*hsp70*,'' respectively, particularly in cases where the specific isoform is not known to the reviewers.

Most prominent in the literature is the HSP70 class, containing a constitutive (Hsc70) as well as a relatively inducible form (Hsp70i a.k.a. Hsp72). The members of the HSP70 class bind to proteins in an energy-dependent (ATP) manner and help to maintain or restore the proper conformation of other proteins in the cell through a repetitive process of binding, bending, and release [Snoeckx et al., 2001]. They also play a role in keeping proteins unfolded until they reach their final destination in the cell or in trafficking irreversibly damaged proteins to lysosomes [Kiang and Tsokos, 1998]. They associate particularly with the cytoskeleton, long-chain fatty acids, and calmodulin.

While the HSP70 proteins play an important role in the unstressed cell chaperoning of nascent proteins, they are particularly useful under stressful situations, where they can have a protective effect and repair damage. ATP hydrolysis is required prior to the release of a refolded protein by Hsp70, indicating that during times of ischemia/hypoxia Hsp70 binding will become more stable, so that misfolded proteins may be kept in a recoverable, semi-folded state until ATP stores recover [Snoeckx et al., 2001]. Mild preconditioning stresses that increase heat shock protein levels, including ischemia-reperfusion and heat shock, have been shown to confer protection against a potentially lethal ischemic insult [Latchman, 2001]. Moreover, transgenic studies that artificially increase the levels of Hsp70 have found an improved resistance to ischemia-reperfusion injuries in vivo [Suzuki et al., 1997; Jayakumar et al., 2001; Tsuchiya et al., 2003]. This protective effect seen on the tissue scale can be due to either the repair of damage as quickly as it occurs on the protein scale, or to the stabilizing influence of Hsp70 binding during periods of low ATP.

Several studies have found that magnetic fields can elevate HSP70 expression or gene activity in cell cultures. Lin et al. [1997] demonstrated a significant increase in expression of heat shock factor (HSF), the transcription factor that regulates HSP70 in HL-60 cells and a rise in Hsp70 itself, following a 8 μT, 60 Hz exposure [Lin et al., 1998]. (Table 1 presents a summary of studies investigating heat shock proteins, ordered by frequency range and then by their discussion in the text.) This increase in Hsp70 expression was also seen in HTB124 cells [Han et al., 1998] and murine H9c2 cells [Carmody et al., 2000]. Interestingly, the rise in protein expression was very quick, within 40 min, and depended on the field strength, with a maximal expression at 8 μT and a significant, but lessened response at 80 μT.

The problems with replication common in the field of bioelectromagnetics are also prevalent here, with a study by Morehouse and Owen [2000] unable to replicate the above results on several exposure systems with different strains of HL-60 cells, including some obtained from the laboratory of R. Goodman and co-workers. While there were several minor procedural differences, such as normalizing the expression of Hsp70 to a housekeeper gene (beta-2-microglobulin), none of them appeared significant. Balcer-Kubiczek et al. [2000] also

TABLE 1. A Summary of Studies Investigating Heat Shock Proteins, Ordered by Frequency Range, (ELF vs. RF) and then by Order of Mention in the Text

Paper	Frequency	Field strength	Exposure	Organism	Assay	Effect
Lin et al. [1998]	60 Hz	8, 80 μT (shielded)	20 min (+20 min rest)	HL-60	Hsp70	Increase
Han et al. [1998]	60 Hz	8 μT	20 min	HTB124	Hsp70	Increase
			3 h continuous			Increase peaked by 2 h
			20 min + 20 min Restimulation			Increase + further Increase w/restim
Carmody et al. [2000]	60 Hz	8 μT	30 min	Murine H9c2 cells	Hsp70	Increase
Morehouse and Owen [2000]	60 Hz	6.3 or 8 μT rms (shielded)	20 min (+10 or 20 min rest)	HL-60	Hsp70	None
Balcer-Kubiczek et al. [2000]	60 Hz (sine and square)	2 mT (shielded)	3 or 24 h	HL-60	HSP70 mRNA	None
Shi et al. [2003]	60 Hz	8 μT (shielded, reintroduced static fields), 100 μT	20 min, 2 or 24 h	HTB124, HL=60, and HaCaT	Hsp70, Hsp 27	None
Tokalov and Gutzeit [2004]	50 Hz	10–100 μT	30 min (+30 min rest)	HL-60	HSP70 mRNA	Increase
		140 μT				None
Pipkin et al. [1999]	60 Hz	100 μT	2 h	HL-60	Hsp70, Hsp27, Hsp90	None
		1 mT				Increase
Miyakoshi et al. [2000]	60 Hz	0.5–50 mT (shielded)	2–20 h	HL-60	Hsp70	None
		50 mT + heat	5 + h			Decrease
Coulton et al. [2004]	50 Hz	20–100 μT	4 h	Leukocytes	HSP27 and HSP70 mRNAs	None
Bodega et al. [2005]	50 Hz	1 mT (rms)	1 h	Cultured rat astroglial cells	Hsp25, Hsp60, Hsp70	None
		Also with a 1 mT DC field	1, 2, or 4 h			None
Tsurita et al. [1999]	50 Hz pulsed	34 mT	3, 6, or 12 h	34i cells	Hsp70, Hsp90	None
			4 h + 42 °C heat			Increase
Malagoli et al. [2004]	50 Hz	300 μT	30 min exposure, repeated with 3 h breaks	Mussel Immunocyte	Hsp70, Hsp90	None
		400 μT				Increase at longer exp.
		600 μT				Increase
Alfieri et al. [2006]	50 Hz	680 μT	24 h	SPAE, HUVEC, CEM, U937 HL-60 cells	Hsp70	Increase
				HUDE WI-38 cells		None
Chow and Tung [2000a,b]	50 Hz	1.2 mT (shielded)	1 h	*E. coli*	DnaK/J	Increase (DnaK)
Kang et al. [1998]	50 Hz	1.5 or 3 mT	20 min (+20 min rest)	34i cells	Hsp70, Hsp90	None
Nakasono and Saiki [2000]	5–100 Hz	7.8–14 mT	6.5 h	*E. coli*	DnaK	None
	50 Hz	14 mT	30 min			None
Czyz et al. [2004a]	50 Hz (“real”)	0.1, 1, 2.3 mT	6 or 48 h	Mouse embryonic stem cells	HSP70 mRNA	None
Junkersdorf et al. [2000]	50 Hz	100 μT (18 °C)	Up to 3 h	*C. elegans*	B-Gal with HSP70 promoter or hsp16	None
		100 μT (30 °C)	2–3 h			Increase
Tipping et al. [1999]	50 Hz	8 μT rms (shielded)	20 min	Fruit flies	HSP70 mRNA	Decrease
		(not shielded)	20 min			None

(*Continued*)

TABLE 1. (*Continued*)

Paper	Frequency	Field strength	Exposure	Organism	Assay	Effect
DiCarlo et al. [2002]	60 Hz	8 μT	1 h (+2 h rest) 30 or 60 min 2× daily for 4 days	Chicken embryos	Hsp70	Increase Decrease
Björåsen et al. [2004]	60 Hz	200 μT	1 h (+4 h rest)	K562 cells	Hsp70	Increase
Miyakawa et al. [2001]	60 Hz	Up to 0.5 T (24–33 °C)	20–120 min	C. elegans	LacZ with hsp16 promoter	Decreased temp. required to induce expression
Leszczynski et al. [2002]	900 MHz GSM	Any SAR of 2 W/kg	1 hr	EA.hy926	Hsp27 expression and phosphorylation	Increase
Shallom et al. [2002]	915 MHz	2.5 W/kg SAR	30 min (+1.5–2.5 h rest)	Chicken embryos	Hsp70	Yes, beginning after 2 h
Ronchi et al. [2004]	0.2–20 MHz	11.4 μT 36.1 μT	2 min/day 10 min/day 15 days each	Rat myocardium	Hsp70	Increase None
Lim et al. [2005]	900 MHz	0.4–3.6 W/kg SAR	20 min, 1, 4 h	Leukocytes	Hsp70, Hsp27	None
Kwee et al. [2001]	960 MHz	2.1 mW/kg SAR	20 min, plus incubations from 0–90 min	AMA cells	Hsp70	Increase (no effect with longer rests)
Czyz et al. [2004b]	1.71 GHz GSM signal	1.5 W/kg 0.4 W/kg 1.5 or 0.4 W/kg	48 h 48 h 6 h	P53-deficient mouse embryonic stem cells	HSP70 mRNA	Increase None None
Lantow et al. [2006]	1800 MHz GSM signal	0.5–2.0 W/kg	1 h	Human Mono Mac 6 and K562 cells	Hsp70	None
Weisbrot et al. [2003]	1900 MHz GSM signal	1.4 W/kg	2 exposures of 60 min each for 10 days	Fruit flies	Hsp70	Increase
Tian et al. [2002]	2.45 GHz	5 W/kg SAR 20–100 W/kg	2–16 h 2–16 h	MO54 cells	Hsp70	None Increase
Cleary et al. [1997]	27 MHz 2450 MHz	25 W/kg 100 W/kg 25 W/kg	2 h exposure with 24 rest period each	CHO and HeLa cells	Hsp70	None None None
Fritze et al. [1997]	900 MHz GSM signal	0.3 or 1.5 W/kg 7.5 W/kg SAR	4 h exposure	Rat brain	HSP70 mRNA	None Slight increase

found no significant effect on HL-60 gene expression following 3 or 24 h of 2 mT sine or square wave 60 Hz magnetic fields. Nor were Shi et al. [2003] able to find an effect on Hsp70 in HTB124 or HL-60 cells exposed to a 60 Hz 8 μT (rms) for 20 min, 2, or 24 h.

Other studies on HL-60 cells with ELF fields have seen promising results, however. Tokalov and Gutzeit [2004] found a somewhat similar dose-response at 50 Hz, with *hsp70* mRNA transcripts expressed for 30 min at 10 μT, a maximal response around 40–80 μT, and no response at higher field intensities (140 μT). The magnetic field exposure was also able to enhance the HSP response following 43 °C heat stress. Pipkin et al. [1999] found no response from a 2-h 60 Hz exposure at 100 μT, but did see an increase in Hsp70, as well as some other heat shock proteins (Hsp27, Hsp90) at 1 mT. At that field strength, the authors found their coils were heating the incubator and employed a water cooling system to counter that problem. Miyakoshi et al. [2000] did not see an effect of a 60 Hz 50 mT exposure alone after 2–20 h; however, they did find that the 50 mT field reduced the Hsp70 expression caused by heat stress of over 5 h. This effect was not seen at lower field strengths (0.5 or 5 mT), and it did not affect the rate of cell death caused by the prolonged heat stress.

The issue of HSP induction by magnetic fields is no clearer in other experimental systems. Coulton et al. [2004] did not see an effect on either HSP27 or HSP70 mRNAs after a 4-h exposure to a 50 Hz field of 20–100 μT rms in leukocytes. Bodega et al. [2005] found no effect on Hsp25, Hsp60, or Hsp70 from cultured rat astroglial cells exposed to 1 mT (rms) 50 Hz fields for 1, 2, or 4 h, with a 24 h rest period prior to analysis. Kang et al. [1998] also did not see an effect on Hsp70 or Hsp90 levels in 34i cells after a 50 Hz exposure at 1.5 or 3 mT for 20 min. Tsurita et al. [1999] found no effect of a ''bursting'' 50 Hz field at 34 mT on Hsp70 in HBL-100, MCF-7, or HeLa cells. They did see an increase above that produced by heat stress at 42 °C within 4 h, although this increase was not significant in all cell lines. Malagoli et al. [2004] did find an increase in Hsp70 and Hsp90 production from 50 Hz at 600 μT after one, two, or three 30-min exposures separated by 3 h, and at 400 μT from the longer two exposures, although no effect was seen at 300 μT. Alfieri et al. [2006] found a cell-line dependent increase in Hsp70 after a 24 h exposure to 50 Hz 680 μT fields, with SPAE, HUVEC, CEM, U937, and HL-60 cells demonstrating an increase in Hsp70, and HUDE and WI-38 cells showing no response. With further examinations using their SPAE cell line, the authors found both exposure-time and field-strength dependent effects (a response at 680 and 300 μT, no significant differences at 150 and 55 μT; the 680 μT field strength differences were not significant with a 4 h exposure, but were with 8–48 h). Interestingly, they did not find a corresponding increase in *hsp70* mRNA, suggesting that the increase in protein levels was perhaps due to an increase in protein longevity, rather than synthesis. In bacterial systems, the homologue of Hsp70, DnaK, has been found to increase after 1 h of 50 Hz fields at 1.2 mT [Chow and Tung, 2000a,b], but not at other field strengths or frequencies: 5–100 Hz at 7.8–14 mT for 6.5 h, with decreasing field strength at increasing frequency, nor at 14 mT, 50 Hz for 30 min [Nakasono and Saiki, 2000].

A number of confounding factors could underlie some of these differing results. For example, Czyz et al. [2004a] found that embryonic stem cells responded to a 2.3 mT ''real'' 50 Hz exposure differently, depending on whether the p53 tumor suppressor gene had been knocked out, as well as on the amount of time elapsed between exposure and analysis. However, while there were selective effects for certain regulatory mRNAs, no effect was seen on HSP70 mRNA for any combination of parameters. Implicit in their design was another factor: the use of a complex waveform that contained frequency components beyond that of a simple 50 Hz sine wave.

In animal models, positive effects have been slightly more plentiful. Junkersdorf et al. [2000] found that when a transgenic reporter gene (β-gal) was under the control of the fruit fly HSP70 promoter in a *C. elegans* host, 50 Hz magnetic fields at 100 μT increased the production of the marker when the nematode worms were heat stressed. However, no effect was seen from the magnetic field alone. A decrease in HSP70 mRNA expression was seen after a 20 min exposure of 50 Hz at 8 μT (rms) for fruit flies reared inside Mu metal boxes, although no effect was seen in larvae reared in ambient fields [Tipping et al., 1999]. Interestingly, they also found that RNA for a housekeeper gene, histone 1.9, also decreased. From the experiments of DiCarlo et al. on chick embryos exposed to UV, the protection conferred by the magnetic fields appeared to influence free radical scavenging. Indeed, heat shock protein 70 has been suggested as the potential protective agent in the chick embryos, and in further studies using ELF fields [DiCarlo et al., 2002] as well as microwaves (915 MHz, SAR of ~1.75 or 2.5 W/kg) [Shallom et al., 2002], it was found that increased Hsp70 levels were associated with the protection against hypoxia. The intensity of the microwave exposure did induce some tissue heating, but positive controls at the same temperature (~39 °C) did not show as much Hsp70 expression. While the similar experiment by Björåsen et al. [2004] could not detect Hsp70 in the chick embryos with their antibodies, they did find that Hsp70 was induced by a 60 Hz, 200 μT field after 1 h (with a 4 h recovery period) in K562 cells.

Much research has also been conducted at radiofrequencies. Ronchi et al. [2004] found an increase in Hsp70 from broadband fields at 11.4 μT, but not at 36.1 μT, despite the fact that there were potential heating effects from the higher exposure (discussed earlier). No effect was seen on Hsp70 or Hsp27 in human leukocytes at 900 MHz for various SARs (0.4–3.6 W/kg) for short durations [20 min, 1 or 4 h; Lim et al., 2005], although Kwee et al. [2001] did see significant increases in Hsp70 in AMA cells exposed to even lower SARs at 960 MHz (2.1 mW/kg for 20 min). Weisbrot et al. [2003] saw a significant increase in Hsp70 expression in *Drosophila melanogaster* with a 1900 MHz GSM signal (1.4 W/kg SAR) at 60 min twice per day exposure over 10 days. Czyz et al. [2004b] found an increase in HSP70 mRNA with a 48 h exposure to a GSM signal (217 Hz modulated 1.71 GHz 1.5 W/kg SAR) in embryonic stem cells that were p53 deficient. No effect was seen in wild-type cells, or in any cells with a 6 h exposure or a 0.4 W/kg SAR. Lantow et al. [2006] did not see an effect on Hsp70 expression in Mac 6 and K562 cells after 1800 MHz GSM exposures at SARs of 0.5–2 W/kg. Tian et al. [2002], found an increase of Hsp70 at very high SARs in MO54 cells exposed to 2.45 GHz fields, but no effect in SARs considered safe for daily exposures (<5 W/kg). Even at similarly high SARs (25 or 100 W/kg), Cleary et al. [1997] did not see Hsp70 production in CHO or HeLa cells 24 h following exposure to 27 or 2450 MHz radiation. Fritze et al. [1997] found a small increase in HSP70 mRNA following a 4 h exposure to ∼900 MHz GSM signal at 7.5 W/kg SAR, but not at lower SARs. In an experiment designed to mimic magnetic resonance imaging procedures, Guisasola et al. [2002] did not find Hsp70 or Hsp27 activation resulting from strong static fields, switched gradients, or RF exposures.

The small heat shock proteins are also widely studied, particularly HSP27 in human tissues, its counterpart HSP25 in rodents, and the nematode homologue, HSP16. These small heat shock proteins are constitutively expressed, as well as possessing some degree of inducibility when the cell is stressed. They form oligomeric units that have hollow, globular shapes [Haslbeck, 2002]; other proteins may bind to the exterior of this globular unit for structural stabilization until refolding can be accomplished. The phosphorylation state of Hsp27 is important for the proper functioning of the heat shock protein, particularly in the way that it forms oligomers and gains chaperone activity. The small HSPs will bind a large range of unfolded proteins, both during times of stress as well as partially folded intermediates during protein synthesis. These bound complexes are very stable, which means that Hsp27 is able to bind to inactivated proteins and hold them in a recoverable state until normal physiological conditions are restored, and refolding can be performed by other chaperones such as Hsp70. Actin, in particular, has been found to be protected by the small HSPs [Snoeckx et al., 2001], which is important not only for the cytoskeleton of all cells, but also for the sarcomeres in cardiac tissue. In addition to preconditioning studies, which tend to induce a variety of protective stress proteins, transgenic studies in vitro [Martin et al., 1997; Brar et al., 1999; Vander Heide, 2001] have demonstrated that increasing levels of Hsp25/27 prior to simulated ischemia-reperfusion injuries can have a protective effect.

Miyakawa et al. [2001] found that the temperature required for a given expression of a reporter gene (*lacZ*) under HSP16 promoter control in transgenic nematode worms was reduced after exposure to a 60 Hz magnetic field at up to 0.5 T. Leszczynski et al. [2002] saw an increase in protein phosphorylation and Hsp27 expression after exposure to a 900 MHz GSM signal. Other reports, discussed previously, have found magnetic field influences on Hsp16 activity in nematodes [Junkersdorf et al., 2000], as well as Hsp27 in cell cultures [Pipkin et al., 1999; Tokalov and Gutzeit, 2004]. Controversy exists for studies of HSP27 as well, and no effect was seen in several papers [Guisasola et al., 2002; Shi et al., 2003; Coulton et al., 2004; Lim et al., 2005].

Currently, there is no clear consensus arising from the number of studies on heat shock proteins and magnetic field exposures. Many studies have reported a dose effect, with differing outcomes depending on exposure duration or field strength. Some of the studies reporting no effects used exposure parameters that one would not expect to produce an effect, based on the effective dose regimes determined by other researchers. However, some more-or-less direct replication attempts have failed (c.f. Morehouse and Owen [2000] attempt to reproduce the work of Goodman and co-workers).

Some caution must be taken in interpreting the results of radiofrequency studies, as there is often the possibility for local heating effects to give false positives. This issue was recently raised at the COST281 workshop, as an abstract by de Pomerai et al. [2004] that called into question the reproducibility of their previous reports of reporter gene activity under the control of the nematode HSP16 promoter after microwave exposure [Daniells et al., 1998; de Pomerai et al., 2000]. The exposure setup used had a power loss that was heating the samples, and when it was recalibrated, the authors could not reproduce their previous findings. What is not clear, however, is why the slight temperature rise seen from their previously configured setup was not sufficient to induce reporter

gene activity when heat alone was given. Perhaps a synergy between stressors is in effect.

Further confusion arises from the inconsistent use of Mu metal shielding for certain exposure setups. Geomagnetic fields have been shown to influence animal behavior and other systems [see Prato et al., 2005 for example] thus it is possible that shielding influences the heat shock/protective response to magnetic fields and becomes another variable for dosing. While the data is not shown, Henderson et al. [2003] described ''extremely high levels of both Hsp60 and Hsp70'' expression in negative control cultures when Mu metal shielding was used. This change was attributed to poor air circulation, but presumably some attempt at engineering an air exchange was made, raising the question as to whether there may have been an effect of the shielding or an interaction of the shielding with the hypoxia. Tipping et al. [1999] found that there was a differential response to a 50 Hz field in fruit flies, depending on whether the larvae were raised in shielded or ambient conditions.

Interactions between some magnetic field exposure conditions and other stressors, for example heat, have also been suggested as a necessary condition for magnetic fields to influence the HSP response [Gutzeit, 2001]. This would suggest that magnetic fields act as a weak stressor, which on their own cannot necessarily induce a heat shock response. That opens the question of magnetic field side-effects: if cells produce stress proteins following a magnetic field exposure, does that imply that the magnetic field is stressful? Or, do magnetic fields ''fool'' the cellular machinery into producing protective proteins when no damage has occurred, perhaps by acting on a downstream part of the signal transduction chain? That open question underlies many studies that use HSPs as markers of stress and is beyond the scope of this review. In the case of using magnetic fields to induce the production of protective proteins prior to a reperfusion insult, both of these points are somewhat moot, as a costress (hypoxia) will be present for the magnetic fields to act upon to amplify the stress response. And if magnetic fields do cause cellular damage, in the short term that is likely to be far outweighed by the potential benefits to the ischemic tissue from cross-protection, resulting in a net protective effect.

The different cell lines and animal tissues introduce further complications. Normally, the levels and relative inducibility of heat shock proteins is tissue, age, and species-dependent [Locke and Tanguay, 1996; Snoeckx et al., 2001]. Often, the so-called inducible form of Hsp70 is found at detectable levels in certain tissues, such as the heart, while the constitutive form also exhibits some degree of inducibility. However, cerebral tissue tends to have a vastly subdued heat shock response [Snoeckx et al., 2001], which may impact the ability of magnetic fields to protect brain tissue from ischemic stroke. Furthermore, the sensitivity of the HSP response to EM fields may have a genetic component [DiCarlo and Litovitz, 1999; Lin et al., 2001], which may explain the different results obtained by different studies. Indeed, a specific electromagnetic responsive element, separate from the heat sensitive domain, has been described in the promoter region of the *HSP70* gene by Lin et al. [1999]. Since transgenic studies indicate that increased levels of Hsp70 are sufficient for reducing the damage following ischemic injuries, the potential for magnetic fields to increase Hsp70 has very promising medical applications. In fact, some patents have been filed to use magnetic fields to induce HSPs for cardioprotection (e.g., R. Goodman et al., US Patent application #20020173691).

MECHANISMS: OPIOIDS

Opioid receptors are found in three basic subtypes, known as δ, κ, and μ. They have been most studied in the brain, where their stimulation has potent analgesic effects. However, they do play important roles in other tissues, such as in the heart, adrenals, and digestive tract. Opioid receptors act as G-protein-coupled receptors that typically influence potassium or calcium channels.

Several experiments have shown that opioids may have protective effects in ischemia-reperfusion injuries, particularly in the heart [Schultz and Gross, 2001]. Furthermore, opioids may protect against damage if administered during the window of opportunity after ischemia sets in but prior to reperfusion [Chang et al., 2005]. Blocking opioid receptors with naloxone (a general opioid antagonist) also attenuated the protective effect of heat stress preconditioning in a study [Patel et al., 2002], further supporting the role opioids may play in ischemia-reperfusion protection. Note that the different opioid receptor subtypes appear to have different functions in this regard. Generally, activating the δ and κ subtypes is considered protective, whereas the μ-opioid receptors can be harmful. Since the heart has a very small number of μ-opioid receptors (if any), opioid activation of the predominantly δ and κ subtypes leads to a protective effect. However, the brain is rich in μ-opioid receptors, so using naloxone to block μ-opioid activation in the brain is protective there [Liao et al., 2003].

For cardiac cells, a potassium-channel mechanism, specifically, the mitochondrial K_{ATP} channel, initiated by the δ-opioid subtype, is believed to be responsible for the protective effect [Schultz and Gross,

2001]. When opened, the K_{ATP} channels will likely reduce the ability of the cardiac myocyte to contract, which will also help prevent calcium overloading, allowing the cell to conserve its energy stores and recover more quickly following the ischemic event. Reducing the anaerobic metabolism may also help reduce the number of metabolic byproducts that may react with an oxygen influx upon reperfusion to produce free radicals. In vascular tissue, K_{ATP} channels may act as vasodilators, improving blood flow.

The use of magnetic fields to treat pain is widely studied, and a number of experiments have examined the effect on opioids in particular. Thomas et al. [1997] found that a specific, pulsed, extremely low frequency magnetic field with a peak field of 100 μT induced antinociception in the land snail and that this was partly due to μ and δ opioid receptor stimulation. Similar pulsed field exposures have been investigated by this group and have been similarly found to induce opioid-related analgesic effects in snails [Thomas et al., 1998], mice [Shupak et al., 2004a], and humans [Shupak et al., 2004b, 2006, in press]. Shielding the ambient magnetic field with a Mu metal box was also found to induce opioid-related analgesia in mice, although this effect took several days of 1 h exposures to manifest itself [Prato et al., 2005]. A single, short exposure to a shielded box did, however, reduce stress-induced analgesia [Del Seppia et al., 2000; Choleris et al., 2002].

Sinusoidal magnetic fields have also been found to influence opioids. Jeong et al. [2000] used a 60 Hz magnetic field at up to 2 mT for 24 h and found a circadian rhythm-dependent effect on pain in mice, with increased nociception at night for fields as low as 500 μT, but only at 2 mT during the day. A long-term study with rats exposed to 50 Hz fields at 5 and 100 μT for 8 h/day, 5 days/week, for 8 months found changes in the expression of opioid receptors in the brain, with differing changes in different regions, for example, an increase in μ-opioid receptors in the hippocampus, and a decrease in the frontal cortex at 5 μT [Zecca et al., 1998].

Perhaps most relevant to the case of cardiac protection, an in vitro study by Ventura et al. [2000] using pulsed ELF fields found an increase in endogenous opioid production. The 1.74 mT peak 50 Hz triangular wave was applied for 1, 4, or 8 h, and all time points had significant increases in prodynorphin mRNA, a δ opioid receptor agonist, with maximal expression at 4 h.

MECHANISMS: NITRIC OXIDE

Nitric oxide (NO) is a reactive gas, able to freely diffuse through cell membranes. It is a common signaling molecule, with a number of physiological roles such as a neurotransmitter, vasodilator, and anti-inflammatory agent. Due to its reactivity and an inability to be stored in vesicles, it is produced on demand by nitric oxide synthases in the body from L-arginine. There are three types of synthase: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). The synthases are controlled by several inputs, notably including calcium/calmodulin, HSP90 binding, as well as a number of protein kinases such as ERK1 and 2 [Wu, 2002].

During ischemia of the heart, NO synthesis is diminished [Jugdutt, 2002]. Restoring physiological levels of NO is generally considered protective, and the use of NO donors, particularly nitroglycerin, in ischema-reperfusion injuries is quite common clinically. There are several potential mechanisms behind this protection, including an increase in vasodilation, which may improve collateral blood flow, and a decrease in leukocyte adherence/inflammatory responses.

However, as NO is a reactive molecule, too much of it can have unintended consequences. It will react with superoxide radicals to produce peroxynitrite ($ONOO^-$) [Jugdutt, 2002]. While this may help reduce the surge of free oxygen radicals produced during the reperfusion stage, it will also remove the NO needed for signaling purposes, and peroxynitrite may itself be cytotoxic. Thus the effect of nitric oxide during ischemia-reperfusion may vary, depending on the state of the cell and the concentration of NO.

Since the brain contains an additional nitric oxide synthase (nNOS) and since the presence of NO may trigger neural firing, which would consume valuable metabolic resources during ischemia, increasing NO is not generally protective for ischemic strokes. Indeed, NOS inhibitors have been found to have protective effects in cerebral ischemia [Ding-Zhou et al., 2002]. Note that timing is also important, as inhibiting NOS prior to ischemia had a damaging effect. That is, an early increase in NO is beneficial, while just prior to reperfusion, it is desirable to inhibit the burst of NO that may result.

Due to the different dynamics of the various nitric oxide synthases, eNOS is generally considered to be protective, as it produces steady amounts of NO at low concentrations, while nNOS and iNOS can produce bursts of higher concentrations, which may be damaging [Albrecht et al., 2003]. There was a differential effect from using L-arginine, a NO donor, in mice that were iNOS deficient as compared to wild-type mice, reinforcing the view that iNOS stimulation may not be beneficial [Zhao et al., 2003].

A number of studies have proposed an effect of magnetic fields on nitric oxide [such as Noda et al., 2000; Yoshikawa et al., 2000] or proposed that nitric oxide is important for the effect of magnetic fields on other systems, for example, analgesic responses [Kavaliers et al., 1998]. Thus, it has been studied extensively and deserves a separate treatment. For more on nitric oxide and how it may be influenced by magnetic fields, see the review by McKay et al. [in press].

MECHANISMS: BLOOD FLOW CHANGES

When an artery is blocked, starving a region of tissue of blood flow, other neighboring blood vessels may be recruited to service the ischemic region. This increased collateral flow may help salvage tissue in the ischemic penumbra, reducing the overall clinical impact. Treatments utilizing this mechanism rely on the overlap of capillary beds originating from different vessels. However, there usually remains an ischemic core that will become necrotic tissue if the blood flow through the occluded vessel cannot be restored (see Fig. 1A).

Another form of collateral flow involves anastomoses, or linkages between major blood vessels (see Fig. 1B). These linkages can allow blood flow to bypass a blockage, and continue to feed tissue downstream of the blockage. Additionally, these anastomoses may improve the access of thrombolytic agents to clots, particularly to the distal area of the clot via retrograde filling. However, these linkages are poorly studied and are not always present in human subjects [Liebeskind, 2003].

Collateral flow may be an adaptive response to lack of flow, and sufficient collateral flow to prevent infarction may only occur in a minority of patients [Wustmann et al., 2003]. However, in patients with some degree of cardiovascular disease, vascular remodeling may occur to increase the effectiveness of anastomoses.

Magnetic fields may aid in increasing blood flow through these collateral channels to potentially reduce infarct sizes. Xu et al. [2000] found that a 50 Hz magnetic field at 1 mT or a static one at 1 or 10 mT could increase blood velocity in a mouse leg muscle. Fields of only 0.3 mT had no significant effect.

Conversely, decreasing blood flow may also be desirable in certain situations, such as in hemorrhagic stroke or in an attempt to ease ischemic tissue into a reperfusion phase (by applying a magnetic field to reduce blood flow as a blockage is removed, followed by one to increase blood flow). Mayrovitz and Groseclose [2005] used contact magnets (approximately 88 mT at the site of blood flow measurements) on the fingers of human volunteers for 15 min, which reduced blood perfusion in the exposed finger relative to an unexposed finger. Morris and Salak [2005] found that a 70 mT static field had a biphasic effect in rat muscle arterioles, tending to bring the tone (i.e., dilation) back to some median value. These findings support the earlier experiment by Okano et al. [1999], where noradrenalin and acetylcholine were used to artificially increase or decrease vasoconstriction, and a 1 mT static field, applied for 10 min, reversed the effect of each drug.

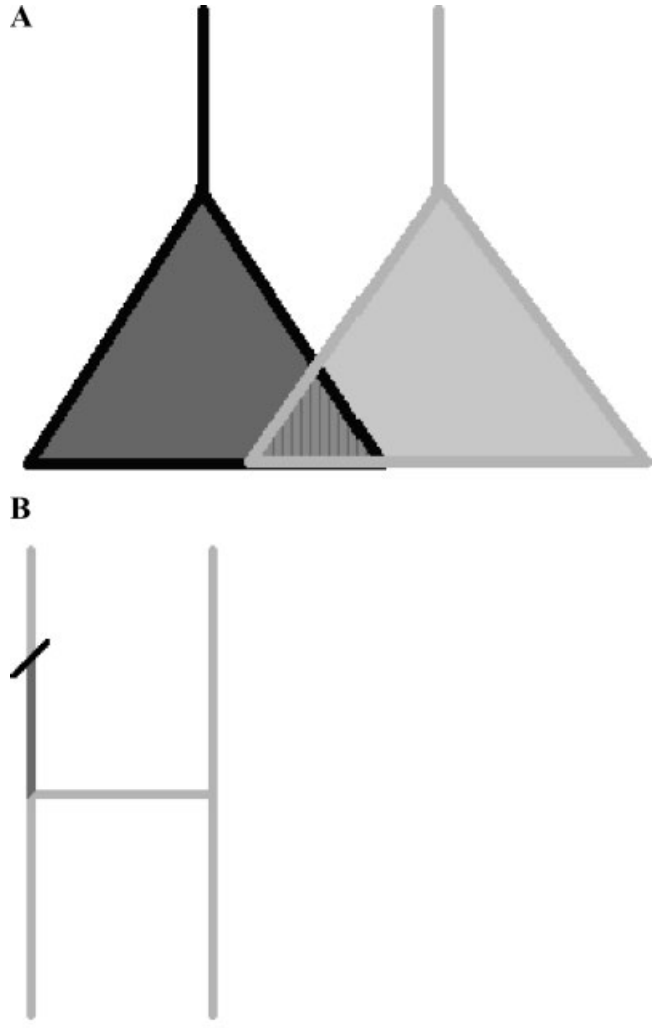

Fig. 1. **A**: A schematic representation of two blood vessels feeding partially overlapping microcirculation beds. If a blockage occurs in the left vessel, the dark gray area will be completely devoid of blood flow and form the ischemic core. The striped area will be partially fed from the right vessel, and form the ischemic penumbra. Increased blood flow from the right vessel may be able to salvage tissue in this zone. **B**: A schematic diagram of an anastomose between large blood vessels allowing blood to bypass a blockage (black mark). Direction of blood flow is from top to bottom.

For a more thorough examination of the interaction between magnetic fields and blood flow, please see the review by McKay et al. [in press].

REPAIRING THE DAMAGE

Following an ischemia-reperfusion injury, there will inevitably be some permanent damage to repair. Magnetic field therapies may play a role in this stage of injuries as well. Much research has been conducted on bone and wound healing with magnetic fields, and some of this may transfer over to internal organs (for a review, see Shupak et al., 2003).

Stem cell therapy is a promising method of regenerating damaged tissue. However, current methods are experiencing poor yields in terms of cells that migrate to damaged areas and then differentiate into a desired cell type. A new study by Ventura et al. [2005] has demonstrated the ability of 0.8 mT (rms) 50 Hz magnetic fields to influence stem cell differentiation, increasing the number of cells that become myocardial cells. Using electric field stimulation Sauer et al. [1999] increased stem cell differentiation into cardiomyocytes via a reactive oxygen species mechanism.

Particularly important for cardiovascular disease would be the creation of new blood vessels, angiogenesis. An in vitro study by Yen-Patton et al. [1988] found that pulsed magnetic fields induced cellular changes indicative of angiogenesis, in a surprisingly short amount of time (as little as 5 h).

DISCUSSION

There are a number of mechanisms by which tissue may become damaged during ischemia-reperfusion. Due to the sudden influx of oxygen and the resulting burst of free radicals produced upon reperfusion, there exists a window of opportunity where intervention may be able to salvage ischemic tissue.

A number of mechanisms for protection have been shown through various studies to have the ability to protect the heart or brain from damage; however conventional treatments often suffer from certain drawbacks: Most obvious is that delivery of blood-borne pharmaceuticals will be, by definition, impaired from the lack of blood flow. Thus magnetic fields may have a particularly useful application, as they can be delivered remotely to activate endogenous protective mechanisms on a cellular level.

Taken individually, the evidence for magnetic fields having cardio- and/or neuroprotective effects via any individual mechanism is, frankly, underwhelming and controversial. However, collectively, the potential influence of magnetic fields on heat shock proteins, opioids, nitric oxide, and collateral blood flow is promising and certainly warrants further investigation into developing protective treatments for ischemia-reperfusion injuries based around electromagnetic fields. This is especially true, given the advantages of noninvasiveness, ease of application, and relatively low cost inherent to electromagnetic field treatments.

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