Heat stress in livestock—the role of the gut in its aetiology and apotential role for betaine in its alleviation

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Summary

A new paradigm for the aetiology of heat stroke in humans has emerged recently, which invokes the possibility of novel approaches to the treatment and prevention of heat stress in livestock species. Exposure to heat results in redistribution of blood to the periphery and compensatory reduction in the blood supply to the gut, which damages cells lining the gut, permitting endotoxin to enter the body. Endotoxin causes tissue damage and an acute–phase immune response. When blood supply resumes, reactive oxygen species and cytokines are released and cause multiple organ injury. Available evidence from livestock species is concordant with this paradigm. The possibility that the integrity of the gut lining is critical to resilience against hyperthermia has great significance for livestock species because energy–dense production diets are known to damage the gut lining. Betaine would impact beneficially at several critical points in the progression of thermally induced tissue damage. These include amelioration of damage to gut and liver tissue, and protection against the effects of endotoxin. It is proposed that administration of betaine to livestock would not only ameliorate losses in heat–stressed animals but also render them more resilient to heat.

Keywords: heat stress, hyperthermia, heat stroke, livestock, betaine

Introduction

Previous reviews have established that heat load has a considerable impact on the productivity and welfare of livestock (Collier *et al.* 1982; Turner 1984; Finch 1986; Sanchez *et al.* 1994; Silanikove 2000; Lowe *et al.* 2002). Others have reviewed strategies for mitigating heat load in livestock (Beede and Collier 1986; Huber *et al.* 1994; West 1999; Kadzere *et al.* 2002; West 2003). Surprisingly, none of these reviews explains the exact mechanism through which hyperthermia exerts its adverse effects. Although veterinary textbooks variously ascribe heat stroke to impairment of cellular function, nervous system activity, or respiratory and circulatory collapse (Robinson 1992; Radostits *et al.* 2000), they shed little further light on the aetiology of hyperthermia.

A new paradigm has recently emerged in the field of human medicine, which places damage to the tissues of the gut as the pivot through which the adverse effects of heat load are promulgated. This invokes the possibility of novel approaches to the treatment and prevention of heat stress. The purpose of this communication is to review the evidence for this paradigm, assess whether it is applicable to livestock species and examine the potential of betaine to constrain or prevent the evolution of reactions critical to the aetiology of heat stress.

Emerging paradigms in the aetiology of hyperthermia

For most of the twentieth century, heat stroke was thought to be a consequence of either direct thermal damage to the brain or circulatory failure (Hubbard *et al.* 1997). While these theories are consistent with the sudden rise in body temperature and drop in blood pressure that precede collapse from heat stroke, and with other symptoms such as delirium, convulsions and coma, they do not adequately explain all of the pathophysiologic changes that are observed in such cases. Pathologies such as a systemic elevation of inflammatory cytokines, widely disseminated intravascular blood coagulation, kidney failure and injury to the liver and pancreas are more consistent with multiple organ failure and sepsis (Majno and Joris 1994) than with brain damage or acute circulatory failure. Two of the pathologies least consistent with these theories are haemorrhage of the gut mucosa and peritoneum, and liver necrosis (Bynum *et al.* 1977; Bouchama and Knochel 2002).

The temporal nature of the effects of hyperthermia is another aspect that is not easily explained by past theories: tissue injury and intravascular blood coagulation continue to develop even after normal body

temperature is restored (De Galan and Hoekstra 1995; Bouchama and Knochel 2002). In livestock species, there is often a delay of three to four days between exposure to heat load and a decrease in production or heat stroke (Maust *et al*. 1972; Hungerford 1990; Hahn 1999). In feedlot cattle, after exposure to a high daytime temperature there are sharp peaks in body temperature of up to 3.5°C that last for 1.5 h during the late evening when ambient temperature has decreased to well below maximum values (Lefcourt and Adams 1996).

Eshel *et al*. (2001), who documented the pathophysiology of thermal injury in anaesthetized monkeys and dogs, described the nature and severity of this delayed response. The animals were heated either until cardiac arrest occurred or for a defined time period followed by resuscitation and cooling. The animals that died during hyperthermia showed evidence of minor haemorrhages of the gut mucosa. The resuscitated animals quickly regained normal body temperature and blood pressure after cooling was applied but began to haemorrhage from the rectum 5–20 hours afterwards and died of cardiac arrest. Necropsies revealed large quantities of blood in the gut, diffuse petechial haemorrhages in all viscera, and severe liver damage.

In a recent review of evidence that has accumulated over the past few decades, Bouchama and Knochel (2002) redefined heat stroke as 'a form of hyperthermia associated with a systemic inflammatory response leading to a syndrome of multi–organ dysfunction in which encephalopathy predominates'. While this new concept acknowledges that brain injury and circulatory failure are consequences of hyperthermia, it places damage to the lining of the gut at the hub of a cascade of events—involving leakage of gut–derived endotoxin into the body and an acute systemic inflammatory reaction—that ultimately result in failure of various organs, including the brain. This progression of events begins with a redistribution of blood flow.

Heat load directs blood flow to the skin at the expense of blood supply to the gut

The first active response to heat load in humans is a redirection of blood flow to the skin to dissipate heat using the cooling effect of sweat evaporation. This redistribution of blood is achieved by dilation of the cutaneous vasculature. In order to prevent a fall in blood pressure, a compensatory constriction of the arteries supplying blood to the gut and splanchnic viscera (liver, pancreas and spleen) occurs (Figure 1).

Reduced visceral blood supply damages cells lining the gut

The reduction in visceral blood supply during heat load causes a substantial decrease in the supply of oxygen and nutrients to this tissue and in the removal of waste products from it. This condition is known as ischemia. All cells have metabolic strategies that enable them to cope with limited periods of ischemia but will incur structural damage if ischemia is prolonged. This leads to loss of function and ultimately to cell death (oncosis). This sequence of events is shown in Figure 2.

Some tissues are less tolerant of ischemia than others are. The gut is particularly vulnerable to ischemia because it is one of the most metabolically demanding tissues of the body. During heat load, blood flow to the gut is reduced by 40–50% (Hales 1983). There is now substantial evidence showing that hyperthermia and the reduction in blood supply to the viscera during hyperthermia result in damage to the cells of the gut (Moseley 1994; Hall *et al*. 1999; Eshel *et al*. 2001; Hall *et al*. 2001; Lambert *et al*. 2002). The villi of the intestines and certain regions of the liver are particularly susceptible (Hall *et al*. 1999).

Although it has been a matter of debate, the exact sequence of events that gives rise to compromised gut membrane integrity is now starting to emerge. While Lambert *et al*. (2002) concluded from their study that heat *per se* results in enterocyte necrosis, other evidence, which showed that heat–induced increases in cell permeability were reversible with cooling (Moseley *et al*. 1994), suggests that these changes are initiated at an early stage of hyperthermia. Both of these studies were conducted *in vitro*; an experiment conducted *in vivo* (Hall *et al*. 1999) showed clear evidence of hypoxia in the villi of the intestine and in the liver, implicating a lack of oxygen as the cause. They also showed that energy utilization by the intestines was increased by hyperthermia. They proposed that increased cellular permeability was caused by reactive oxygen species (ROS) generated because of oxidative stress and depletion of ATP.

ROS are a class of powerful oxidants that remove electrons from molecules, initiating destructive chain reactions that impair cell structure and membrane integrity. One of the main ways in which ROS are thought to exert their destructive effects is by impairing cellular ionic homeostasis (Chihuailaf *et al*. 2002). The enzyme, calcium ATPase is responsible for maintaining the intracellular Ca^{++} concentration at a level that is typically 10 000 times less than that outside the cell. This enzyme, however, contains thiol groups, which are susceptible to attack by ROS. Loss of calcium ATPase activity results in an increase in intracellular Ca^{++} which, in turn, activates proteases that attack the cytoskeleton of the cell (Chihuailaf *et al*. 2002).

Damage to cells lining the gut permits entry of endotoxin

Endotoxin is a lipopolysaccharide component of the outer membrane of most gram–negative bacteria that inhabit the gut, and has exceedingly toxic effects when it enters the body. Under normal conditions, the membranes of cells lining the gut are impermeable to endotoxin and the cells themselves are joined together by 'tight junctions', effectively forming a barrier that prevents endotoxin from entering the body. When this

barrier is compromised, endotoxin invades the body. Elevated concentrations of endotoxin have been detected in the portal veins of heat–stressed monkeys, rabbits, in heatstroke victims and in marathon runners (Butkow *et al*. 1984; Gisolfi and Mora, 2000; Jessen 2001). That the increase in blood endotoxin concentrations could be prevented by oral administration of antibiotics (Butkow *et al*. 1984) indicates that the endotoxin originates from the lumen of the gut. A noteworthy aspect of this report is that endotoxemia became evident after only one hour of heat exposure—when body temperature was elevated by as little as 1°C; this suggests that damage to the gut lining occurs at relatively mild degrees of heat stress. This is concordant with results obtained *in vitro* that show that an increase of 1.3°C was sufficient to induce increased permeability of kidney cells (Moseley *et al*. 1994).

The increase in the blood concentration of endotoxin during hyperthermia coincides with an increase in the production of ROS (Hall *et al.* 2001), indicating that ROS play a key role in the destruction of cellular membranes and subsequent ingress of endotoxin. Endotoxin induces the release of a variety of cytokines from the cells of the gut and liver (Malago *et al.* 2002; Black 2003). Cytokines are hormone–like chemical messengers that facilitate communication between cells responsible for the immune response and initiate the acute phase immune response. Under normal conditions, this immune response promotes tissue repair by recruiting host defence mechanisms and facilitating access to the site of tissue damage. Endotoxin, however, stimulates an inappropriate response in which there is an imbalance between inflammatory and anti–inflammatory cytokines. This imbalance may result in either immunosuppression or inflammation injury (Bouchama and Knochel 2002). The inflammatory cytokines, tumour necrosis factor (TNF) and interleukin–1 (IL–1), are expressed at high levels, and are capable of particularly harmful effects. IL–1, for example, induces fever, depresses appetite, stimulates bone resorption and causes capillaries to leak, while TNF causes cell death and blood clotting (Majno and Joris 1994).

Endotoxin initiates reperfusion injury

A significant advance in the understanding of the aetiology of hyperthermia was the finding that the rise in endotoxin levels during hyperthermia could be eliminated by inhibiting the activity of the enzyme, xanthine oxidase (Hall *et al*. 2001). The proteases that are activated by the influx of Ca^{++} ions during ischemia (see Figure 2) also convert the innocuous enzyme, xanthine dehydrogenase, to xanthine oxidase. In the presence of oxygen, xanthine oxidase converts the products of ATP depletion, xanthine and hypoxanthine, to a highly toxic species of ROS, which damages cells.

In the ischemic cell, this enzyme and its substrate merely accumulate since the reaction is constrained by a lack of oxygen. One of the effects of endotoxin is to stimulate the release of nitric oxide (NO), a potent vasodilator. The subsequent resumption of blood flow to the gut (and hence oxygen supply) activates xanthine oxidase, unleashing a tide of ROS. In humans, the magnitude of NO production is proportional to the severity of heat stroke (Alzeer *et al*. 1999). This is known as reperfusion injury (Figure 3), and can be even more destructive than ischemia, since it not only exacerbates damage at the site of the insult but also propagates damage throughout the body.

Endotoxin precipitates septic shock and multiple organ Injury

The endotoxin–mediated release of NO induces vasodilation of the visceral vasculature. Since peripheral vasodilation is also present during heat load, this results in a sharp decrease in blood pressure (Figure 1), provoking an abrupt rise in heart rate and collapse. This is known as septic shock.

Splanchnic vasodilation also facilitates the spread of endotoxin to other tissues of the body where further tissue damage is propagated by cytokine induction. Some of these—the pyrogenic cytokines—act on the thermoregulatory centre of the brain to induce a fever–like state. This is thought to be the reason for the sudden rise in body temperature that precedes collapse from heat stroke. Other cytokines such as TNF and IL–1 are thought to be responsible for systemic damage to other tissues such as the brain, kidneys and lining of the vasculature (Bouchama and Knochel 2002), and provide an explanation for the observation that heat–induced tissue damage often persists after body temperature has been returned to normal. A synopsis of these events is presented in Figure 4.

The heat shock response

The body responds to a variety of insults that damage intracellular proteins by producing heat shock proteins. These 'molecular chaperones' stabilize proteins, protecting them against denaturation, and re–fold denatured proteins, restoring their functionality (Kregel, 2002). Exposure to heat induces production of heat shock protein–70 (HSP–70) in the gut, liver and other tissues (Hotchkiss *et al*. 1993; Flanagan *et al*. 1995). HSP–70 attenuates heat–induced increases in membrane permeability (Moseley *et al*. 1994) and increases the resilience of cells to endotoxin (Hotchkiss *et al*. 1993; Chi and Mestril 1996; Chen *et al*. 2001) and ROS (Marini *et al*. 1996). In addition, heat shock proteins inhibit the production of pro–inflammatory cytokines, up–regulate the expression of anti–inflammatory cytokines and induce protective growth factor responses that promote cell–healing (Malago *et al*. 2002). It has also been suggested that heat shock proteins assist with the maintenance of intracellular ion pumps by transporting key proteins (Moseley, 1994).

The beneficial effects of HSP are so numerous that it is not surprising that the productive life of Holstein

cattle is associated with promoter variants in the gene that encodes HSP–70 expression (Schwerin *et al*. 2003). It has even been suggested that the development of methods for promoting the accumulation of HSP–70 in cells would enable expansion of the normal human thermoregulatory zone to 41°C and above, and improve the success of organ and tissue transplantation (Gisolfi and Mora, 2000). Several studies have demonstrated that induction of HSP prior to heat exposure confers increased resilience against hyperthermia (Wischmeyer *et al*. 2001; Kregel, 2002; Wischmeyer, 2002; Zulkifli *et al*. 2003). This evidence confirms that protein denaturation is a critical component of heat injury. The finding that the greatest increase in HSP concentrations following exposure to heat occurs in the tissue of the liver and gut (Flanagan *et al*. 1995) provides additional support for the contention that damage to the cells of the gut is central to the adverse effects of heat load.

Relevance of the new paradigm to livestock species

Most of the empirical evidence for the critical role of gut permeability in the aetiology of heat stress was derived from studies that used rodents as experimental models. While this paradigm has been the subject of considerable research effort in the medical field over the past decade, the relevance of these findings to livestock species has received little attention. Recently, it was shown that administration of the antioxidant, ascorbic acid, to heat–stressed poultry attenuated the increase in HSP–70 in heart tissue (Mahmoud *et al*. 2004). Although gut tissue was not examined in this study, it does at least suggest that hyperthermia is associated with increased ROS production in this species.

In humans exposed to heat load, ischemia of the gut and liver represents the first step in a cascade of events that precipitates endotoxemia, reperfusion injury and ultimately, multiple organ failure. Gut ischemia is a consequence of increased diversion of blood to the skin, since evaporation of sweat is the main conduit for heat dissipation in humans. It is pertinent to examine whether blood flow to the gut is affected by heat load in livestock species, since the contribution of evaporation from the skin to heat dissipation is less in some species than in others.

Heat loss from sweating is greatest in the horse, followed, in descending order, by cattle, sheep, pigs and poultry (Reece, 1991). Birds do not have sweat glands and dissipate heat by panting and gular flutter, a rapid oscillation of the floor of the mouth and upper part of the throat (Schmidt–Nielsen 1990). Pigs, which do not sweat much and pant ineffectively due to their relatively small mouths, rely on the evaporative cooling effect of moisture acquired by wallowing in water. Sheep do sweat, but make considerable use of panting to dissipate heat. Sweating is the major form of heat dissipation in cattle (Cunningham, 1992). Sheep sweat to a limited extent and rely to a considerable extent on panting to dissipate excess body heat by evaporation of moisture from the tongue and surfaces of the nasal cavity. The frequency of respiration in sheep increases from 50 breaths/min to 320 breaths/min following exposure to heat load, and blood flow to the respiratory muscles consequently increases by up to ten–fold (Hales 1983; Sakurada and Hales 1998). Blood flow to the nasal mucosa and sublingual salivary gland increases four–fold (Sakurada and Hales 1998). Although blood flow to the skin of the torso does not increase during heat load (Hales 1983; Sakurada and Hales 1998), blood flow through arteriovenous anastomoses in the extremities (limbs and ears) is substantially increased (Hales 1983; Jessen and Feistkorn 1983; Sakurada and Hales 1998). Anastomoses are connections between the arterial and venous sides of the vascular bed that allow blood to bypass the capillaries, diverting a greater proportion to the surface veins. Blood flow to the abdominal organs, stomach and ileum is decreased by 55%, 58%, and 32% respectively (Sakurada and Hales 1998).

Gut permeability does not appear to have been measured in hyperthermic livestock species. There is, however, no reason to believe that gut epithelial cells of livestock would be any more tolerant to a lack of oxygen than those of other species are. Since the magnitude of heat–induced visceral ischemia in the sheep exceeds that observed in the human (40%; Hales 1983) and is similar to that required to permit entry of gut endotoxin in pigs (50%; Fink *et al*. 1991), it is reasonable to assume that hyperthermia would result in damage to the cells of the digestive tract of livestock species. On a macroscopic level, evidence of congestion of the mucous membranes of the intestine in cattle that died of heatstroke (Terui *et al*. 1980) indicates that hyperthermia does result in damage to the gut in this species. Furthermore, their observation of 'infiltration of small round cells in the lamina propria of the small intestine' resembles the 'cellular vacuolization' and 'intracellular swelling' that accompanied an increase in permeability of gut epithelium in the studies of Lambert *et al*. (2002) with hyperthermic rats. Khogali *et al*. (1983) reported 'severe congestion of most serosal surfaces and of the organs' in sheep that had died of heat stroke. The loss of bowel movements in hyperthermic cattle (Terui *et al*. 1980) is suggestive of gut damage; identical symptoms have been observed in other species (Eshel *et al*. 2001).

Although it seems probable that gut permeability in hyperthermic ruminants would be compromised to a similar extent as in monogastric species, it is possible that the consequences of this would be more severe since the rumen is populated with bacteria that are predominantly of the endotoxin–producing gram–negative type. While there do not appear to be any studies that have attempted to measure endotoxin concentrations in hyperthermic livestock, Sakurada and

Hales (1998) found that indomethacin treatment reduced the rise in body temperature of sheep exposed to heat stress. Since indomethacin blocks prostaglandin pathways involved in endotoxin–induced fever, this infers that endotoxin was, indeed, present.

It has been established that intravenous injection with rumen bacterial endotoxin induces severe endotoxic shock in calves (Nagaraja *et al*. 1979). The pathology that was observed in these endotoxic calves included haemorrhages and disseminated intravascular coagulation—symptoms that have also been observed in hyperthermic cattle (Terui *et al*. 1980) and sheep (Hales, 1983; Mustafa *et al*. 1984). Smith (2001) asserts, 'Disseminated intravascular coagulation, renal failure, and myocardial necrosis are frequent complications' [of hyperthermia in livestock species]. This pathology has also been observed in hyperthermic humans, monkeys and dogs (Khogali and Mustafa 1984; Eshel *et al*. 2001) and, as discussed previously, is consistent with the concept of tissue injury consequent to endotoxin entry from the gut.

The possibility of reperfusion injury does not appear to have been studied in livestock species exposed to heat load. We recently measured the concentrations of HSP–70 in leukocytes taken from cattle exposed to a cyclic heat load protocol for a period of five days (Cronjé, Alley and Pegg, unpublished). Although there was a modest elevation of HSP concentrations during the heat load phase, a far greater increase occurred subsequent to heat exposure (Figure 5). This is, as far as we are aware, the first demonstration of a delayed *in vivo* HSP response to hyperthermia in any species. Since it has been established that HSP concentrations are elevated by reperfusion following ischemia (Bedirli *et al*. 2004), we propose that the increase in HSP concentrations that we observed following hyperthermia is indicative of tissue injury caused by reperfusion of the gut.

Significance of the new paradigm for livestock systems

The possibility that the integrity of the gut lining is critical to resilience against hyperthermia has great significance for livestock species that are fed energy–dense production diets. Stomach ulcers are common in intensively managed pigs and are associated with finely ground pelleted diets (Eisemann and Argenzio 1999a). Although the aetiology of these lesions has not been clearly elucidated, it has been shown that they are associated with increased intracellular ROS production in the gastric mucosa cells (Eisemann and Argenzio 1999b). Since thermally induced gut tissue damage is also associated with ROS production, pigs fed such diets would be more susceptible to the tissue–damaging effect of hyperthermia. Rumen acidosis is common in feedlot and dairy animals fed diets that contain high levels of grain. Considerable quantities of endotoxin accumulate in the rumen under conditions conductive conducive to the development of acidosis (Huber 1976). Furthermore, acidosis damages the rumen epithelium. Grain–based diets can double the osmolality of rumen digesta, leading to a rise in the osmotic pressure gradient between the gut circulation and the rumen contents. This results in rapid movement of water from the blood across the rumen epithelium, causing epithelial cells to separate from the basement membrane. The resulting necrosis affords endotoxin and bacteria entry to the body (Nagaraja and Chengappa 1998; Owens *et al*. 1998). Liver abscesses in feedlot cattle are a direct result of the entry of endotoxin and bacteria through a damaged rumen epithelial lining (Owens *et al*. 1998). The high incidence of liver abscesses in feedlot cattle (22%; Smith 1998) suggests that these animals would be particularly vulnerable to hyperthermia. Although ruminants reduce their feed intake during hot weather, passage rate through the digestive tract is also reduced; the net result is that rumen acid production per unit of feed increases and rumen pH decreases (Sanchez *et al*. 1994). In addition to this, acidosis is associated with an increased level of lactic acid in the blood and dehydration (Elam, 1976); both these conditions would reduce resilience to heat stress.

Although no empirical work appears to have been done to link acidosis with susceptibility to hyperthermia in cattle, the following comment of Elam (1976) is worthy of note: 'The highest incidence of acidosis and similar problems is observed in feedlots during the warmer seasons, and this is especially high during the summer months'. Disruption of feeding patterns by changes in the weather has been implicated as a cause of acidosis (Owens *et al*. 1998), and feedlot deaths 'related to digestive causes' have been reported to peak during the summer months (Miles *et al*. 1998). Suppression of feed intake during a heat wave and engorgement during a subsequent cool period is thought to have been responsible for an incident in which a large number of feedlot cattle died of hyperthermia during a second heat wave that followed the cool period (Hahn 1999). Although Hahn's interpretation was that the additional heat load associated with the prior consumption of food exceeded the animals ability to dissipate it, this is not particularly convincing since five days elapsed between the first opportunity they would have had to engorge themselves and their deaths. In addition, the heat wave in which the cattle died was milder than the first and would not normally be regarded as life threatening. A scenario in which ischemic injury to the gut (caused by the first heat wave) is followed by reperfusion injury (initiated in consequence of restoration of normal blood circulation to the gut during the cooler event) is a more attractive hypothesis.

The problems associated with feedlot diets can be mitigated by reducing the energy content of the diet or the amount fed, and this has been advocated as a strategy for managing animals during heat waves (e.g., Mader *et al*. 2002; Davis *et al*. 2003). This practice,

however, results in a loss of income due to slower growth rates and the cost of mixing and storing additional feed ingredients. There is also an increased risk of deaths due to acidosis and bloat upon re–feeding. Such contingency plans have not been widely adopted by industry because the accuracy and predictive value of weather forecasts and thermal–humidity indices falls short of what is required to assess the relative value of these trade–offs. Solutions that aim to increase the resilience of animals to heat load are far more likely to find acceptance with industry. Recent advances in our understanding of the aetiology of hyperthermia invoke the possibility of improving resilience against heat load by manipulating gut membrane integrity.

Strategies for increasing resilience to heat load

The main events in the progression of thermal injury (Figure 4) represent potential points at which interventions could be implemented to prevent or ameliorate the adverse effects of heat load. Inhibition of the vasodilatory response would be counter–productive, since this would diminish the rate of heat dissipation from the body. On the other hand, blood pressure would drop if visceral vasoconstriction were attenuated. It would thus appear that the presence of visceral ischemia must be accepted as an inevitable consequence of exposure to heat load. However, several agents exist that could be employed to confer greater resilience against the effects of ischemia. In 1996, Gaffin and Hubbard published a comprehensive review of prophylactic and therapeutic agents that have potential utility for the prevention and treatment of heat stress and heat stroke in humans. These include anti–endotoxin agents, anti–cytokine agents, potassium channel agents and dietary omega–3 fatty acids. With the exception of the latter, the use of these agents in livestock that produce products destined for human consumption is unlikely to be acceptable for food safety reasons. Betaine is an agent that could be administered to livestock with safety and ease. Betaine is a tri–methyl derivative of the amino acid, glycine, and is present in the cells of micro–organisms, plants and animals. In the past, it was most commonly thought of as either an osmolyte or a methyl–donor (Kidd *et al*. 1997; Craig 2004). More recently, it has been described as a chemical chaperone, since it repairs denatured proteins and interacts with molecular chaperones, the heat shock proteins. In addition to these attributes, it has several other properties that make it a most attractive candidate for attenuating thermal tissue damage.

Betaine and dehydration

In addition to ion pump activity, many cells also make use of organic osmolytes to regulate cell volume. Betaine is selectively absorbed or secreted by a wide variety of cells including those of the gut, liver and kidney (Sheikh–Hamad *et al*. 1994; Kettunen *et al*. 2001a, b). *In vitro* studies using gut tissues from poultry chicks supplemented with betaine showed that it alters the movement of water across the intestinal epithelium during a hyperosmotic challenge (Kettunen *et al*. 2001c). Skin cells also accumulate and release betaine in response to extracellular osmotic challenges (Warskulat *et al*. 2004). The osmotic properties of betaine may have a beneficial impact at several points in the prevention of dehydration during exposure to hot environments:

- Reduction of increased vascular permeability and consequent loss of blood plasma water that has been observed in cases of hyperthermia (Matthew *et al*. 2000);
- Reduction of epidermal dehydration in animals that sweat;
- Reduction of the decrease in kidney function that is often observed in cases of hyperthermia (Terui *et al*. 1980; De Galan and Hoekstra 1995); this would increase the efficiency of electrolyte and water retention and prevent generalized dehydration.

Betaine and gut tissue damage

Histological evidence shows that dietary betaine supplementation alters the morphology of the cells lining the small intestine of poultry and stabilizes the structure of the gut mucosa (Kettunen *et al*. 2001b). In this trial, these changes were of sufficient magnitude to afford protection against damage caused by intestinal parasite infection. Other studies with poultry have shown that betaine reduces the invasiveness of coccidia, decreases the incidence of coccidia–related gut lesions, ameliorates the decrease in gut villus height associated with coccidiosis and enhances the phagocytosis of coccidia by gut macrophages (Augustine *et al*. 1997; Augustine and Danforth 1999; Klasing *et al*. 2002). In addition to the implication that a reduction in parasite–induced damage to gut cells would make animals less susceptible to heat–induced cell damage, there are several other ways in which betaine could ameliorate thermal damage to gut cells.

- Reduction of osmotic damage to gut tissue.

  As discussed previously, the consumption of diets containing high levels of finely ground grain is likely to predispose animals to thermally induced damage to gut tissue. Betaine accumulation by these tissues would make them less susceptible to the damaging effects of the elevated osmolarity of gut contents associated with such diets and would thus render them less susceptible to thermally induced damage to gut tissue.

- Reduction of the intracellular Na^+ accumulation.

  Cell membranes are highly permeable to water.

Movement of water into or out of the cell is driven almost exclusively by osmotic gradients. An increase in the concentration of ions within the cell leads to an influx of water and cellular swelling within minutes. Conversely, cells shrink when the extracellular fluid becomes hyperosmotic. The importance of ion pumps in maintaining an osmotic gradient compatible with normal cell volume is apparent when one considers the vast differences in the concentrations of osmotically active substances between the inside and outside of cells (Table 1). It is thought that the reason why enzymes and other intracellular biological processes require a medium that is high in K^+ and Mg^{++} is that cells first evolved in a primordial sea that was rich in these elements (Granner 1993). Cellular hydration, and hence cell volume, exerts profound influences on cell function. Many metabolic processes are sensitive to small changes in cell volume; these include protein turnover, metabolism of amino acids, ammonia, carbohydrates and fatty acids, membrane transport and endocytosis (Häussinger 1996). Intracellular ion pumps are damaged during hyperthermia. In this situation, the osmotic properties of betaine may help to prevent cell swelling and the associated entry of Ca^{++} that activates enzymes that destroy cell membranes.

Table 1 Relative ionic composition of the extracellular and intracellular environment in mammals (adapted from Granner 1993).

Ion	Relative concentration	
	Outside	Inside
Ca^{++}	25 000	1
Cl^-	25	1
Na^+	14	1
HCO_3^-	2.7	1
K^+	1	35
PO_4^{3-}	1	30
Mg^{++}	1	20

- Reduction of intracellular energy depletion.

  The initial event in the progression of effects that results in increased membrane permeability during hyperthermic ischemia of the gut is depletion of cellular energy reserves. Ion pumps consume the major part of intracellular energy; the Na^+/K^+ pump alone accounts for over 50% of energy consumption in the cells of the intestine in cattle fed concentrate diets (Huntington 1999). This is perhaps not surprising when one considers that one million Na^+ ions are pumped out of the cell per second (Haines 2001). The cost of maintenance of a favourable intracellular ionic environment is thus of the order of 300 000 ATP molecules per second.

  Incubation of erythrocyte cell membranes with betaine resulted in a decrease of 64% in the activity of the Na^+/K^+ pump and a decrease of 73% in Ca^{++} pump activity (Moeckel *et al*. 2002). Although the exact mechanism underlying these effects is unclear, they were beneficial since a mixture of osmolytes that included betaine increased the resilience of cells to hypo–osmotic conditions by 42%. Inhibition of gut ion–pump activity of this magnitude would save a considerable amount of energy. A 64% decrease in Na^+/K^+ activity alone would decrease the total energy consumption of the intestine by 32%. In the heat–stressed animal, a reduction of gut energy expenditure could delay or even abrogate the cascade of events that is initiated by depletion of intracellular energy reserves.

  Because the gastrointestinal tract accounts for 25% of energy consumption of the body (Huntington 1999), a reduction in Na^+/K^+ pump activity of the magnitude referred to above would be equivalent to a whole–body energy saving of 8%. Results showing that dietary betaine supplementation reduced the maintenance requirements of growing pigs by 5.5% (Schrama *et al*. 2003) illustrate the practical impact of the effect of betaine on the energy costs associated with ion pumping.

- Reduction of protein denaturation.

  As a consequence of the reduced efficacy of ion pumps in the gut of the hyperthermic animal, increased concentrations of Ca^{++} activate proteases that denature proteins (Figure 2). Since proteins are essential to the functional integrity of the cell membrane, this initiates a vicious cycle in which membrane permeability is progressively increased. In the process of denaturation, proteins uncoil, losing the spatial configuration that determines their specific functional activity and exposing buried side–chains that cause them to bind together in aggregates. Diamant *et al*. (2001) showed that mitochondrial protein was irreversibly inactivated by heat treatment at 44°C within 15 minutes. Remarkably, addition of betaine to the medium fully protected the protein from denaturation over the entire 40–minute duration of the trial. The amount of betaine in *Escherichia coli* cells was also correlated with a reduction in heat–induced protein aggregation. Cells normally respond to thermal insults by synthesizing heat shock proteins that restore the functionality of denatured proteins by refolding them and preventing them from aggregating. In the trial mentioned above, betaine increased the rate at which heat shock proteins refolded proteins by up to 50% and the rate at which proteins were disaggregated by 2.5–fold. Sheikh–Hamad *et al*. (1994) observed a three–fold reduction in

thermally induced heat shock protein expression in canine kidney cells that had been treated to accumulate betaine. They concluded that betaine had protected intracellular proteins to such an extent that the signal for heat shock protein induction was attenuated.

The failure of addition of betaine to the incubation medium of heat–exposed avian fibroblast cells to elicit a heat shock protein response (Petronini *et al*. 1993) illustrates an important point: the transporter that enables cells to take up betaine is induced by extracellular hyperosmolarity but not by heat (Sheikh–Hamad *et al*. 1994). The corollary to this is that in order to be effective in protecting livestock from heat exposure, betaine should be fed prior to thermal events when feed intake and osmotic stress on the cells lining the lumen of the gut are at their highest and not only during heat waves when feed intake is reduced. Given that the occurrence of heat waves is unpredictable, the best strategy for protection of livestock would be to commence the addition of betaine to the feed with the onset of the warm season, and to continue until all danger of heat waves is past.

Betaine and ischemia–reperfusion injury

In addition to its protein–stabilizing effect, betaine also appears to modulate the function of certain types of liver cells during ischemia–reperfusion. The addition of betaine to the medium used to perfuse intact rat livers, which were subject to ischemia and then re–oxygenated, decreased the production of lactate dehydrogenase and aspartate transaminase—indicators of liver damage—by 50% and 67% respectively (Wettstein and Häussinger 1997). The increase in vascular resistance—an index of microcirculatory disturbances—was 57% lower in the presence of betaine. When the cells were perfused with hyperosmolar media, the release of tumour necrosis factor–α was increased; addition of a mixture of betaine and taurine suppressed the release of this cytokine. From this, it was concluded that the protective effects of betaine in their experimental model were not due to protein stabilization but due to modulation of Kupffer cell function. Kupffer cells are the resident macrophages of the liver and have an immune function.

Betaine and endotoxin injury

The final event in the cascade of insults that precipitates heat stroke is the entry of endotoxin from the gut into the circulation. Kupffer cells remove endotoxin from the systemic circulation, but they also release potentially harmful quantities of reactive oxygen species, nitric oxide and inflammatory cytokines such as TNF–α into the circulation when endotoxin is presented in large amounts.

Kim and Kim (2002) supplemented the drinking water of rats with betaine for two weeks prior to injecting them with endotoxin. They found that betaine reduced serum concentrations of aspartate aminotransferase, alanine aminotransferase and bilirubin—indices of liver damage—following the endotoxin challenge, indicating that betaine exerted a strong protective effect on the liver. They also observed that the endotoxin–induced elevation of serum TNF–α concentration was decreased by 38% by betaine supplementation and that of nitric oxide by 21%. These results indicate that betaine protects animals from the adverse effects of endotoxin by inhibiting Kupffer cell activation.

Summary of the effects of betaine

Betaine has a diverse range of beneficial effects on cellular metabolism, many of which would have beneficial effects on reactions that are critical to the propagation of thermal tissue damage. These are summarized in Figure 6.

Conclusions

Sufficient evidence exists to validate the new paradigm for the aetiology of hyperthermia. While there is some equanimity on the relative importance of possible effectors of increased cell membrane permeability, there is widespread agreement in medical research circles that damage to the gut plays a central role in exacerbating the adverse effects of hyperthermia. Although there is a paucity of data from livestock species, the evidence that exists is consistent with this paradigm. Betaine is used widely throughout the livestock industry, but for reasons related to its effect as a methyl–donor and osmolyte. There is conclusive evidence that betaine also exerts a range of beneficial effects on stressed or damaged cells. The synthesis of this review is that a considerable number of the actions of betaine would impact beneficially at critical points in the progression of thermally induced tissue damage. It is proposed that administration of betaine to livestock would not only ameliorate losses in heat–stressed animals but, if given prior to thermal events, would render them more resilient to it. Unpublished results of trials commissioned by industry are encouraging. Betaine appears to improve mortality rate and feed conversion ratio in chickens exposed to heat stress (Danisco Animal Nutrition), and improve feed intake, body temperature and respiration rate in heat stressed feedlot steers (Feedworks Pty Ltd.).

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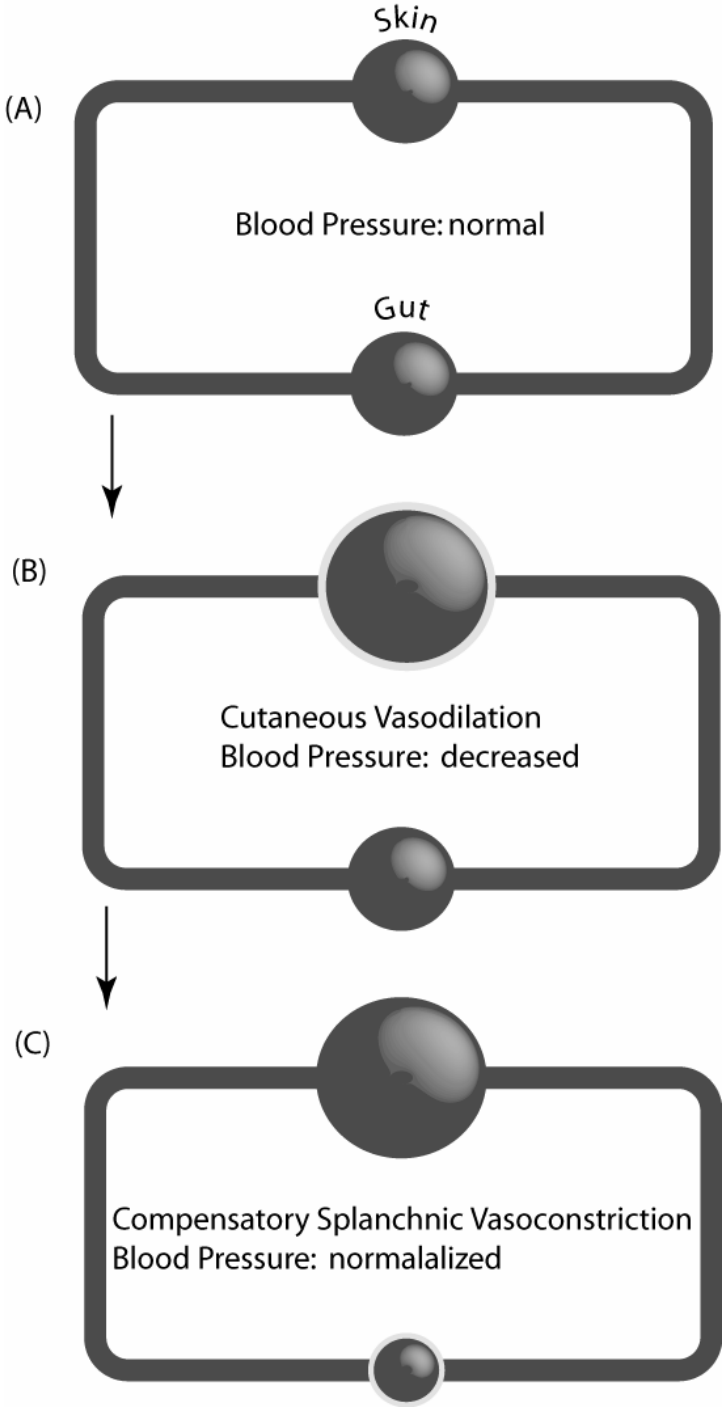

Figure 1 The heart maintains pressure within a closed circulatory system that contains a fixed volume of blood (A). Pressure will fall if the volume of any part of the system is increased (B) unless there is a corresponding reduction in the volume of another (C) or an increase in the output rate of the heart. Cardiac output increases during hyperthermia, but is insufficient to counter the effects of cutaneous vasodilation; blood pressure is restored by vasoconstriction of the vessels supplying blood to the gut and liver.

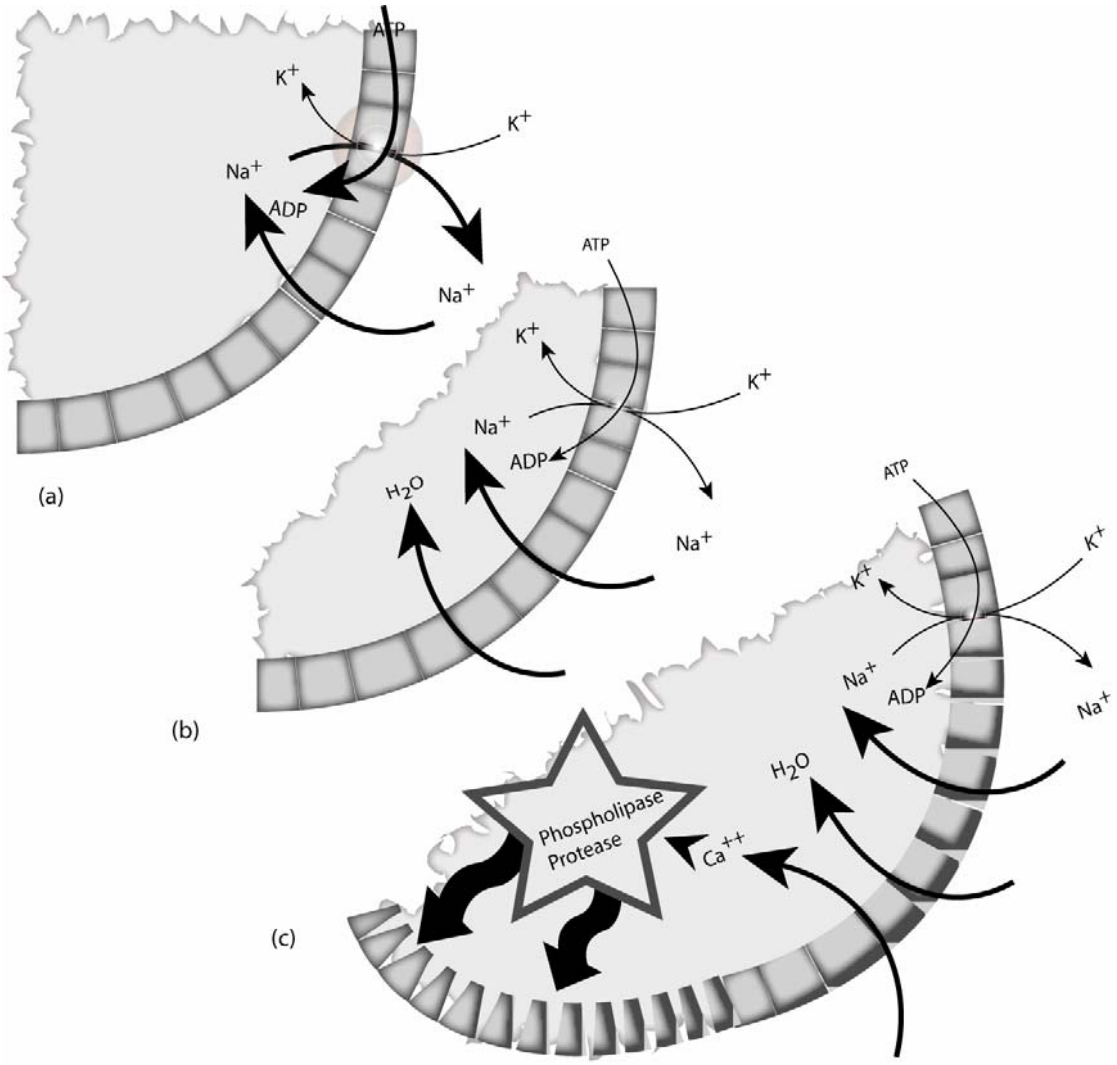

Figure 2 Ischemia and oncosis. Intracellular concentrations of ions such as Na^+, K^+ and Ca^{++} are actively regulated by energy–consuming ATPase driven pumps such as the sodium–potassium pump (a). This pump alone accounts for 33% of the energy expenditure of the cell. During ischemia, a lack of oxygen impairs mitochondrial re–generation of ATP from AMP, and the Na^+/K^+ pump fails to export sufficient sodium from the cell. Sodium accumulates within the cell, and water moves into the cell along the new osmotic gradient (b). Water causes swelling of the cell, stretching the cell membrane and allowing Ca^{++} to enter the cell. In addition, the ability of Ca^{++} ATPase pumps to export Ca^{++} from the cell is impaired by ROS, generated as a result of the breakdown of AMP. Calcium accumulates within the cell and activates phospholipase and protease enzymes, both of which break down components of the cell membrane, exacerbating the leakage of ions into the cell (c).

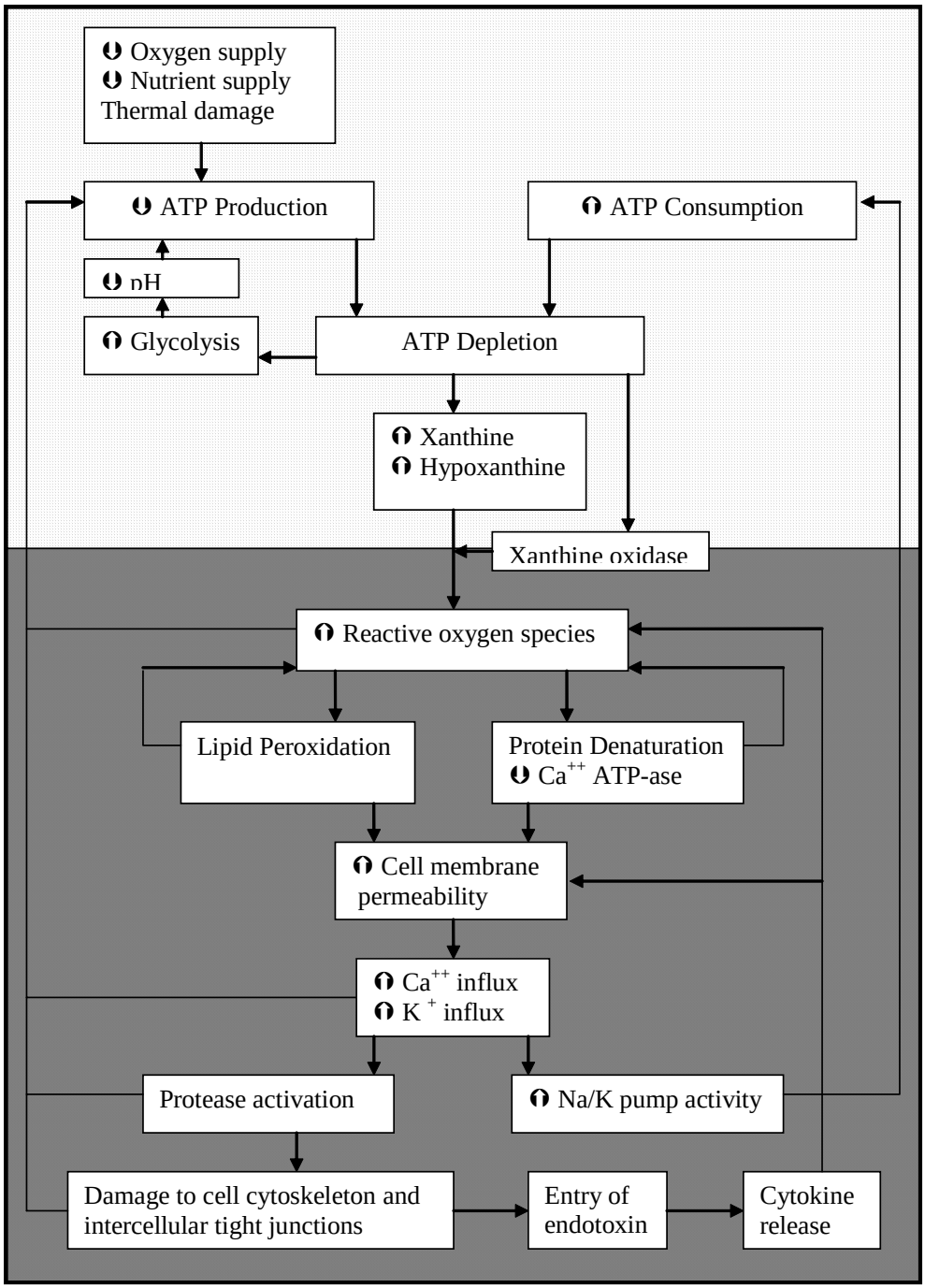

Figure 3 The activation of xanthine oxidase, which accumulates during ischemia (light shading), by reperfusion (dark shading) exacerbates and perpetuates cell damage (increase = ↑; decrease = ↓).

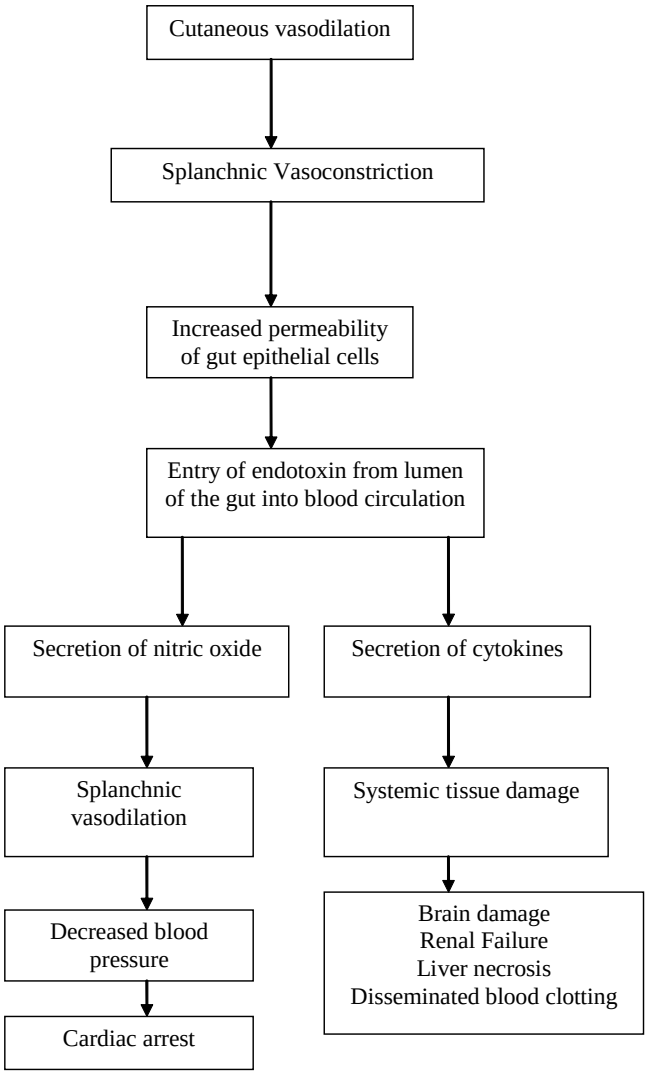

Figure 4 Synopsis of key events during hyperthermia.

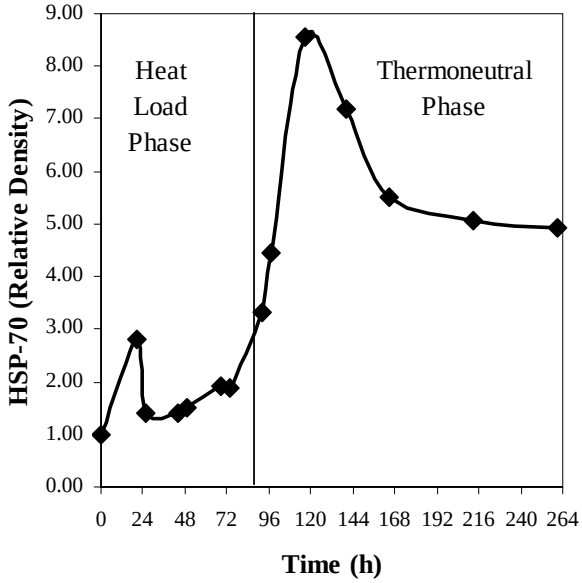

Figure 5 Heat shock protein–70 concentrations in leukocytes from cattle exposed to heat stress. Density was analysed by SDS–PAGE electrophoresis and is expressed relative to that recorded during a thermoneutral phase prior to heat stress.

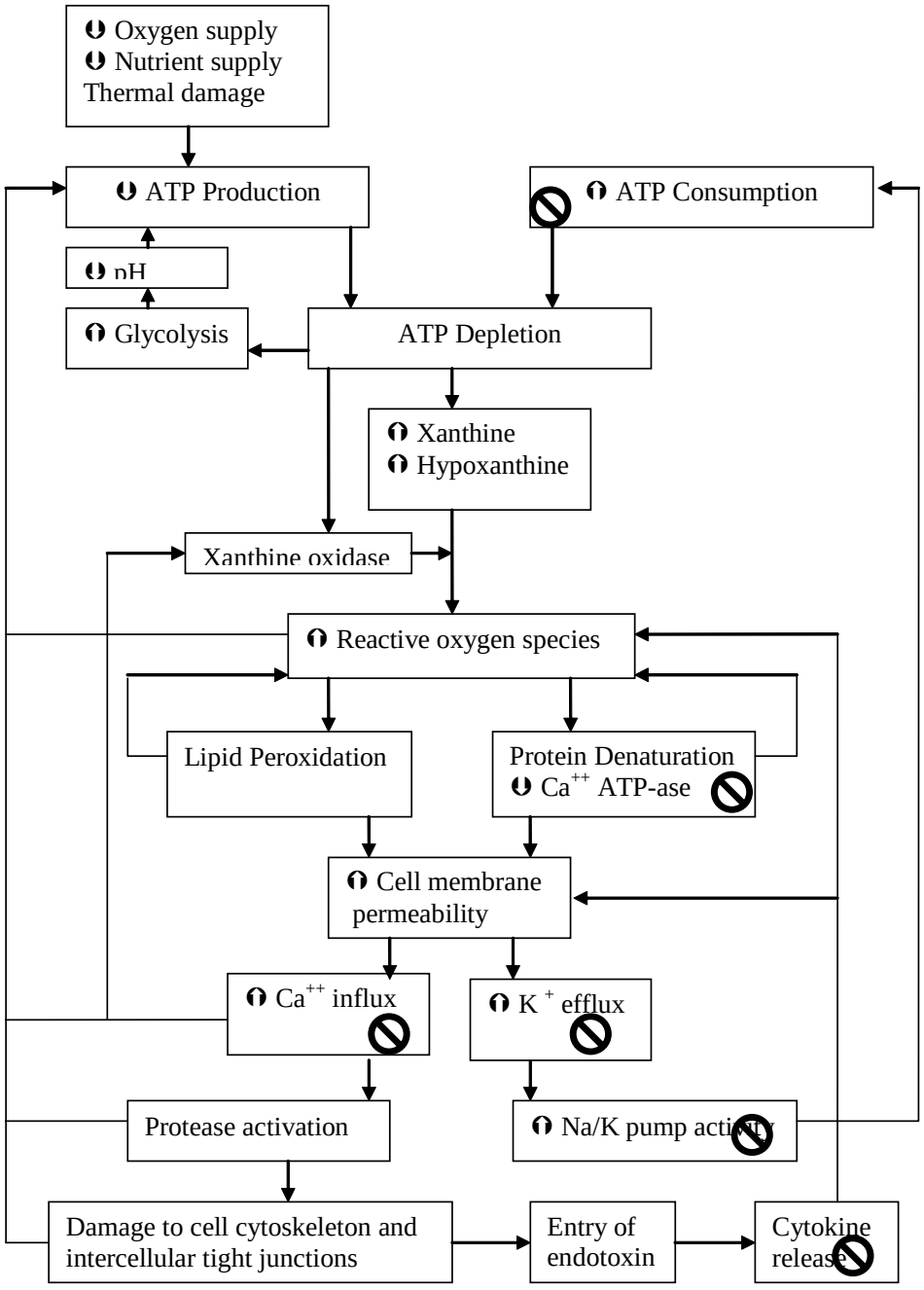

Figure 6 Putative effects of betaine in the aetiology of hyperthermia. Reactions for which there is documentary evidence of an inhibitory effect of betaine (see text) are indicated by the symbol,⊘.