

Expert Opinion

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Trans-sodium crocetin for treating hypoxia/ischemia

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Background: *Trans*-sodium crocetin (TSC) is a novel compound that offers promise as a treatment for conditions caused by hypoxia or ischemia. TSC was originally developed at the University of Virginia for hemorrhagic shock, as part of the battlefield casualty program sponsored by the US Office of Naval Research. Animal toxicology studies have demonstrated that high levels of TSC are well-tolerated, and a Phase I clinical study has shown that TSC is also safe in humans. **Objective:** The drug acts via a mechanism that has not been previously exploited in a pharmaceutical. TSC increases the rate of oxygen diffusion between the erythrocytes and the tissues by altering the 'structure' of water in blood plasma. It does this by causing additional hydrogen bonds to form among the water molecules. **Conclusion:** Further development of TSC for hemorrhagic shock and other hypoxic/ischemic conditions is being done by Diffusion Pharmaceuticals LLC.

Keywords: diffusion, hypoxia, ischemia, oxygen, respiratory, shock, *trans*-sodium crocetin, water structure

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1. Introduction

Trans-sodium crocetin (TSC) is the leading drug from Diffusion Pharmaceuticals LLC, Charlottesville, VA, USA. This compound embodies a novel mechanism of action, which was first proposed in the 1970s by Professor John Gainer of the Department of Chemical Engineering at the University of Virginia, and which has recently become better understood.

Many processes, both natural and industrial, involve the movement of substances that will later react with each other. Thus, it is possible for chemical changes to be limited by the rate of transport to the reaction site [1]. Sometimes chemical reactants move to that site by a process known as diffusion, which results from Brownian motion of molecules along a concentration gradient [2]. In making physiological comparisons to other types of reacting systems, Gainer and coworkers concentrated on the movement of oxygen through the blood plasma, first suggesting that the diffusion of oxygen could be altered by varying the plasma composition [3,4]. They also showed that crocetin, a natural carotenoid compound that increases diffusion of oxygen through plasma, was effective as a therapeutic treatment in a rat model of atherosclerosis [5].

Treating animal models of various diseases with crocetin continued until the mid-1990s. In the early 1990s, their research direction had turned to treating rat models of hemorrhagic shock [6]. These studies showed that crocetin increased whole-body oxygen consumption in rats undergoing a 40% hemorrhage. In addition, magnetic resonance spectroscopy (MRS) studies showed that tissue energy stores (as defined by the ratio of ATP to inorganic phosphate) were increased with crocetin. Later studies showed, though, that crocetin was not beneficial when treating more severe (55 – 60%) blood losses [7].

Crocetin is a mixture of different isomers, as are many natural carotenoids. The suspicion that the *trans*-isomer was the important one led to the development

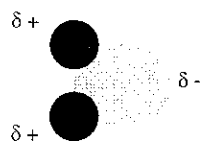


Figure 1. Water molecule showing the negatively charged oxygen atom (gray) and the positively charged hydrogen atoms (black).

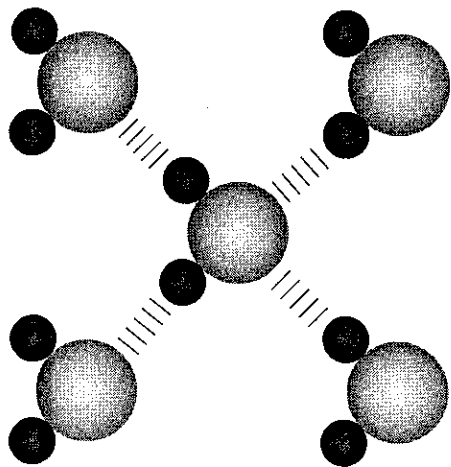


Figure 2. A water molecule can form a maximum of four hydrogen bonds with its neighboring water molecules.

of a pure *trans*-isomer salt compound, which was named *trans*-sodium crocetinate, or TSC. Unlike crocetin, it worked well in the more severe hemorrhagic shock model [7]. Although TSC was first produced using an extraction process, later methods were developed for the chemical synthesis of TSC to good manufacturing practices (GMP) standards.

Although many of the earlier studies with TSC involved the treatment of the ischemic conditions of hemorrhagic shock in both rats [7-10] and swine [11], a few other studies involved treating purely hypoxic situations. For example, one study showed that TSC was capable of promoting survival in rats breathing 10% oxygen [12]. TSC also was able to increase arterial PO_2 values in a rat model of acute respiratory distress syndrome (ARDS) caused by injection of oleic acid [13]. The first patent [14] for *trans*-sodium crocetinate was issued in May 2000 to John Gainer. That patent was assigned to Diffusion Pharmaceuticals LLC after the formation of the company by Gainer and David G Kalergis in 2001.

2. *Trans*-sodium crocetinate for treating hypoxia/ischemia

2.1 Mechanism of action

To fully understand the mechanism of action of TSC, it is helpful to examine the steps involved in the movement of oxygen to or from the erythrocytes. For example, in the latter

pathway, oxygen must become unbound from the hemoglobin, cross the erythrocyte cell membrane, cross the plasma, cross the vascular wall and tissue membranes, and move through the cells to the mitochondria. Those steps might be thought of as a series of resistances, which must be overcome in the pathway of oxygen. A number of studies in the 1970s and 1980s focused on whether or not one of those resistances might be greater than the others and thus dominate the overall process. Diffusion through plasma was found to account for 70 – 90% of the overall resistance [15-17]. Although these are *in vitro* results, rather than *in vivo* ones, they still suggest that an answer to increasing tissue oxygenation could lie in a better understanding of the diffusion of oxygen through blood plasma.

Diffusion follows Fick's Law [2], which dictates that the rate of oxygen diffusing through plasma depends on the plasma thickness, the concentration gradient of oxygen, and a proportionality constant known as the diffusion coefficient, sometimes called the diffusivity. The anatomy of the vascular system sets the plasma thickness; thus, the easiest factor to change might seem to be the concentration gradient.

Increasing the oxygen concentration gradient is frequently done in order to get more oxygen into the bloodstream or into the tissues. For example, one can increase the arterial blood oxygen tension by breathing a gas containing a higher percentage of oxygen than air (air is 21% oxygen). One can also cause more oxygen to migrate to the mitochondria of the tissues by increasing the concentration of oxygen in the plasma with the addition of hemoglobin-like molecules or fluorocarbons, or by causing the erythrocytes to release more of their bound oxygen.

A completely overlooked approach to altering diffusion, however, is to increase the diffusion coefficient. The value of the diffusivity of oxygen through blood plasma reflects the innate ability of oxygen to move through the plasma. Such movement is undoubtedly related to the intermolecular forces that exist among the molecules of the plasma. Since the major constituent of plasma is water, its intermolecular forces are predominately due to hydrogen bonds. Although there are various hypotheses concerning how water molecules are bound together, it may be possible to picture this in a relatively simple manner.

As everyone knows, a water molecule contains two hydrogen atoms and one oxygen atom. Possibly not as familiar is the fact that the hydrogen molecules contain a net positive charge and the oxygen molecule contains a net negative charge. Thus, a water molecule is said to be a dipole since it has opposite charges on each side, as shown in Figure 1. Because negative and positive charges attract one another, water molecules can form bonds with one another, bonds that are called hydrogen bonds. Theoretically, it is thought that any water molecule can form four such hydrogen bonds with the water molecules surrounding it, as shown in Figure 2.

However, it has been discovered that, on average, a water molecule forms fewer than four hydrogen bonds. The literature

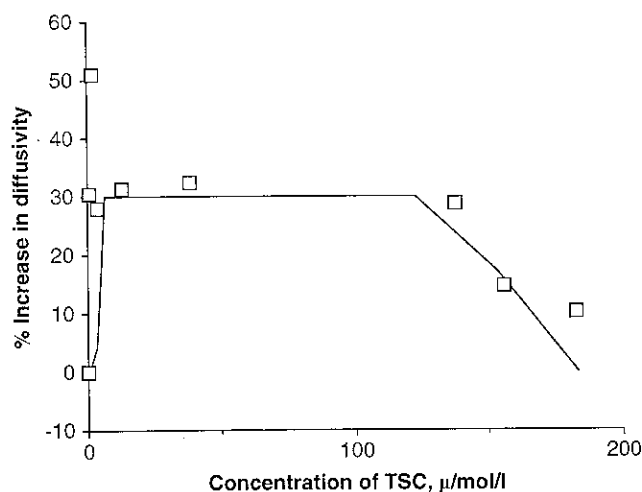


Figure 3. The effect of TSC on the diffusivity of oxygen (line) and glucose (open squares) through water or plasma.

reports different average numbers of hydrogen bonds existing around each water molecule, ranging from 2 to 3.6 [18,19]. Thus, if the number of hydrogen bonds could somehow be increased to a value closer to four, the water molecules would look more like the theoretical picture in Figure 2; that is, the water would become more structured. Conversely, it should also be possible to destroy some of the existing hydrogen bonds, resulting in less structure in the water. Creating more structure (analogous to forming ice from liquid water) causes a reduction in density, while destroying hydrogen bonds (analogous to forming liquid water from ice) causes an increase in density.

It has been shown with computer simulations [20], as well as through physical experimentation [21], that TSC increases water structure and results in additional hydrogen bonds being formed when added to water. This causes a decrease in the plasma density, resulting in a reduction in the resistance offered by the plasma to the diffusion of other molecules through it.

Such a decrease in density of the water would result in an increase in the diffusivity of oxygen or of any small solute through water or plasma. Figure 3 shows the increases in the diffusivity of oxygen (line), as well as glucose (open squares), through plasma or water that occur with the addition of TSC. Although the absolute values of the diffusivity of oxygen through plasma and the diffusivity of glucose through plasma are different, both change by the same percentage when TSC is added.

Since many carotenoids are free-radical scavengers, a study was done to determine if TSC also scavenges radicals [22]. TSC appears to be a very good scavenger of hydroxyl radicals and a lesser one for superoxide anion radicals, but both these effects were statistically significant only at concentrations higher than the dosage levels used in preclinical animal studies [22]. It was also found that other free-radical scavengers do not

increase whole-body oxygen consumption in hemorrhaged rats [22]. Thus, it is not believed that TSC's effects are due to interactions with free radicals but, rather, to its ability to increase the diffusivity through plasma.

Most pharmaceuticals depend on a biochemical mechanism of action. Actions based on physical chemical changes have seldom, if ever, been suggested as the basis for a drug. Although a novel type of mechanism might be surprising to some, the results obtained when using TSC in standard animal models of hypoxia/ischemia lend support for this unique new type of pharmaceutical entity.

2.2 TSC in animal models of ischemia/hypoxia

A number of animal studies have been done to examine the effects of TSC in ischemic or hypoxic models. These studies are described in the following sections and summarized in Tables 1 and 2.

2.2.1 Animal models involving ischemia

The first test for TSC was done in a rat ischemia model, one designed to simulate the treatment of hemorrhagic shock with fluid resuscitation [7]. Fluid resuscitation was, and still is, the usual therapy for hemorrhagic shock. Male rats were placed inside a chamber that measured whole-body oxygen consumption, and oxygen consumption rates before hemorrhage were noted to be around 20 – 25 ml/min/kg.

After removing 57% of the rats' estimated blood volumes via an external syringe pump, the whole-body oxygen consumption rate had fallen to 10 – 15 ml/min/kg. It continued to fall to essentially zero over the following 15 – 20 min in animals given a standard resuscitation fluid of normal saline; however, adding TSC to that same saline fluid resulted in oxygen consumption rates rising to values of 15 – 20 ml/min/kg in 10 – 20 min [7].

The TSC-treated animals all lived, while the untreated rats mostly died. This first study of ischemic shock also showed that, if the rats were given a smaller volume of the TSC infusion fluid, their oxygen consumption values returned to close to normal values, and they were more alert after a few hours [7]. This led to the idea that perhaps extreme blood losses could be treated with administration of less fluid, and that perhaps even single small-volume injections of TSC would work [8].

In the second hemorrhagic shock study, TSC was given as a small bolus injection to rats 3 – 5 min after they had been hemorrhaged of around 60% of their estimated blood volumes [8]. Controls were given similar injections of isotonic saline. Mean blood pressures of all rats averaged around 100 mmHg in the pre-hemorrhage state. After hemorrhage, all of the animals had mean blood pressures around 30 – 35 mmHg. The blood pressures of the control rats continued to slowly decrease, and at 4 h only 1 out of 7 rats was still alive. In the TSC-treated rats, however, the blood pressures began to increase soon after the injection was given and reached a value near 80 mmHg after about 30 min.

Table 1. Ischemia studies.

Condition	Species	Ref.	Summary of results
Hemorrhagic shock	Rat	[7]	TSC added to isotonic saline infusion: Increased oxygen consumption and increased survival
Hemorrhagic shock	Rat	[8]	Single bolus injection of TSC: Increased blood pressure Decreased tachycardia Decreased plasma lactate levels Increased survival rates
Hemorrhagic shock	Rat	[9]	After 20-min delay in treatment, repeated bolus injections of TSC: Increased blood pressure Decreased tachycardia Decreased liver damage Increased survival after 2 bleeds
Hemorrhagic shock	Rat	[10]	Repeated bolus injections of TSC: Reduced TNF- α (liver) Reduced TNF- α and IL-10 (spleen)
Hemorrhagic shock	Swine	[11]	Single bolus injection of TSC: Gave immediate but temporary improvement in oxygen kinetics Prolonged metabolic improvement Increased survival
Ischemic stroke	Rat	[25]	TSC infusion: Reduced infarct volume Increased brain PO ₂ in hypoxic penumbra
Ligated aorta	Rat	[26]	Single bolus injection of TSC (drug was a mixture of isomers): Did not affect the rise in PCO ₂

All 10 of the TSC-treated rats were alive at 4 h. After a severe hemorrhage, the heart rate increases. Such tachycardia occurs in both this rat model and in humans; however, TSC depressed the magnitude of the increase in heart rate.

A lesser hemorrhage of 43% of the estimated blood volume was used in another group of rats (a smaller volume was removed so that they would not die during the experiment), and plasma lactate levels were determined over time. At 90 min after the hemorrhage, the plasma lactate level was 8 mmol/l in the controls, while the TSC-treated animals had a lactate level of 2 – 3 mmol/l, which is not too different from a normal value.

As noted, TSC was given in this study 3 – 5 min after the hemorrhage ended. Obviously, a first responder in an emergency situation probably would not reach a human victim that quickly, so another study [9] was done to determine how a 20-min delay in TSC treatment would affect the recovery of hemorrhaged rats.

In this study, a hemorrhage volume of 60% of the estimated blood volume was used, and it was found that 15 min after the hemorrhage ended the blood pH had dropped from its pre-hemorrhage value of 7.42 to a value of 7.23 [9]. This was supported by base deficit values that increased from values averaging -1 to values around 14. Thus, the animals were extremely acidotic at a time of 15 min post-hemorrhage, and treating them with a single bolus of TSC 5 min later caused a temporary rise in the blood pressure. The TSC bolus injections were then repeated every 10 min over the following hour. After each injection, there was a transient rise in the blood pressure which, after six injections, led to an overall improvement in the rats. One hour later, the blood pH in the TSC-treated rats was back to the pre-hemorrhage value. The saline-treated rats had improved slightly and now had a base excess of 8, while the base excess of the TSC rats was 4 [9].

This study also involved measuring the liver transaminase activity over a period of 24 h. In the control rats, the values of both glutamic-oxalacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) enzymes had doubled by a day after the hemorrhage, indicating that there were necrotic liver cells. In the TSC-treated rats, however, the values of GOT and GPT were unchanged over that same time period. Subsequent histology supported the fact that there were much fewer necrotic liver cells in the TSC-treated animals [23].

A different rat model was used in another study of hemorrhagic shock, one in which blood was removed and the blood pressure maintained at around 40 mmHg for about 30 min before treatment. Again, TSC was dosed by repeated boluses, and cytokine levels were determined [10].

Since inflammatory cytokines have been implicated in mortality and tissue damage, it was thought that perhaps they could be somehow involved in the effect of TSC on hemorrhagic shock. TSC resulted in lower concentrations of TNF- α in the liver and spleen as well as lower concentrations of IL-10 in the spleen. Similar effects on cytokines have been seen with 100% oxygen [24]. Further investigations

Table 2. Hypoxia studies.

Condition	Species	Ref.	Summary of results
10% oxygen	Rat	[12]	Bolus injections of TSC, repeated every 30 min: 100% survival of TSC-treated rats for 3 h of the study 33% survival for controls Decreased base deficit Improved hemodynamic parameters
12% oxygen	Dog	[27]	Single bolus injection of TSC: Did not increase performance in isolated gastrocnemius muscle undergoing contractions
14% oxygen	Dog	[28]	Single bolus injection of TSC: No effect on pulmonary oxygen exchange in exercising dogs
ARDS	Rat	[13]	Bolus injections of TSC, repeated every 10 min for an hour: Increased arterial PO ₂ Decreased arterial PCO ₂
Cancer radiation	Rat	[29]	Five once-daily (pre-irradiation) TSC injections: Increased survival from 25% to 70% Decreased tumor size
Treadmill running	Rat	[30]	Single bolus injection of TSC: Did not affect oxygen consumption in rats during treadmill running

concerning the effect of TSC on a systemic inflammatory response have not been done, but such an effect could possibly be similar to one resulting from oxygen therapy.

The previous rat hemorrhage studies were supported by the United States Office of Naval Research, who also supported another study utilizing TSC in a swine model of combined traumatic brain injury and hemorrhage [11]. This study appeared to support the results from the rat studies, although some of the beneficial changes seen were more transient than in the rat model.

Another ischemic experiment involved a rat stroke model [25]. In this study, two carotid arteries and the middle cerebral artery were occluded. After 2 h, the vessels were reopened, and the animals killed at the end of 24 h. It was found that TSC reduced the infarcted volume by about 60%. In addition, Licox oxygen electrodes placed in the ischemic penumbra of the brain showed that TSC caused a

significant increase in the PO₂ of that portion of the brain in those animals.

A final study using TSC in an ischemic model involved inducing subdiaphragmatic hypotension by intermittently ligating the proximal aorta [26]. A single injection of TSC was found to have no effect on either the degree of PCO₂ elevation or the time to peak PCO₂. As noted in the conclusions of that study, the drug used was not the pure *trans*-isomer [26]. Therefore, the drug actually used was not TSC but more likely a sodium salt of crocetin. Table 1 briefly summarizes all of the ischemia studies, in order to demonstrate the breadth of indications for which TSC may be beneficial.

2.2.2 Animal models not involving ischemia

Some of the preclinical animal studies of TSC have not involved any alteration in blood volume or flow in order to produce hypoxia. Those studies are briefly described below.

One of the first studies with TSC involved determining changes in rats subjected to breathing 10% oxygen [12]. This created a rather severe hypoxia, with only 2/6 control animals surviving for 3 h; however, all 12 TSC-treated animals survived for the same period. TSC also resulted in improved hemodynamic status and a reduction in base deficit.

Two other studies involving dogs breathing reduced oxygen gases did not show any effects when using TSC [27,28]. One study involved the isolated gastrocnemius muscle during isometric contractions while the animal was breathing 12% oxygen [27]. TSC did not improve muscle performance. The second study involved gas exchange in foxhounds breathing 14% oxygen [28]. Again, no differences were seen with the dosing of TSC.

A rat model said to produce ARDS-like symptoms was also studied using TSC [13]. This model involves injecting oleic acid into the femoral vein, which causes arterial PO₂ values to fall to 50 – 60 mmHg after 1 h and PCO₂ values to increase to ~ 50 mmHg. TSC, given as repeated boluses, resulted in an increase in the arterial PO₂ levels and a decrease in the arterial PCO₂ values. While not measured in this study, previous investigations [6] have shown that crocetin, a similar compound, does not affect ventilation or blood flow.

A study published in the spring of 2008 [29] details the use of TSC as a radiosensitizer for cancer. Cancerous tumors are hypoxic, and increasing their oxygen levels is thought to make radiation therapy more effective. This study involved a rat model of glioblastoma multiforme treated with a single dosage of 8 Gy of radiation. Either saline (controls) or TSC was dosed for 4 days prior to radiation, as well as on the day of radiation. The survival rates at 2 months were 25% for the controls and 70% for the TSC-treated animals.

In a final study, TSC was found to not affect oxygen uptake in rats breathing air and running on a treadmill [30]. Table 2 summarizes the studies that involved hypoxia without ischemia.

2.3 Other studies with TSC

TSC has been shown to possess a favorable toxicity profile. Both rats and dogs were tested with dosages that were at least two orders of magnitude larger than the optimal efficacious dose for those species. Such large dosages did not cause adverse effects.

In addition, a Phase I clinical trial was completed in mid-2007 in normal subjects, using single intravenous bolus injections. That trial also showed no adverse effects at TSC dosages greater than the expected efficacious human dosage level. The efficacious human dosage will be determined in clinical studies scheduled for 2008.

2.4 Conclusion

Trans-sodium crocetinate is the leading drug candidate from Diffusion Pharmaceuticals LLC. It is the most developed of their pipeline of small molecules that increase the diffusion of small solutes, such as oxygen, through blood plasma. The increased diffusion occurs because TSC and the other pipeline molecules alter the 'structure' of the water portion of the plasma by increasing the numbers of hydrogen bonds formed among the water molecules.

Much of the preclinical testing has involved the use of TSC for hemorrhagic shock. However, TSC has also shown beneficial effects in other ischemia/hypoxia indications in animal models, especially for treatment of respiratory disorders, such as ARDS [13], and as a sensitizer for radiation of cancerous tumors [29]. Ischemic stroke represents another promising indication but more studies are needed. Diffusion Pharmaceuticals intends to further develop TSC and its other drugs for these and other conditions in which hypoxia, whether related to an ischemic insult or not, is a major factor.

3. Expert opinion

Many of the ischemic/hypoxia animal models show improvement with TSC, particularly for treatment of hemorrhagic shock, respiratory disease and ischemic stroke, and as a radiosensitizer for cancer. Some studies have not shown a beneficial effect but that may be due to the drug or dosage used. As noted previously, one of those studies did not use the pure *trans*-isomer of TSC [26]. Two of the studies [27,28] involved giving a rat dosage (the only dosage known at that time) to dogs. More recent studies indicate that an efficacious dosage (mg/kg) for a dog is approximately four times that for the rat, suggesting that these studies should be repeated with an increased TSC dosage.

Diffusion Pharmaceuticals LLC is developing a therapeutic agent based on its ability to alter water structure. The mechanism by which TSC appears to work has not, apparently, been considered previously, and it may seem surprising to those familiar with the more common biochemical or genetic mechanisms of action. This may be at least partly due to changes that have occurred over the past 30 years. Research concerning physiological change has gone from broader

systemic considerations to therapies that act at the molecular level, either from a receptor or genetic point of view. For example, research dedicated to discovering a limiting resistance in the movement of oxygen in and out of the bloodstream was done in the 1970s and 1980s by several research groups but has not been done since. This is not to say that receptor and genetic approaches are not important or useful, but simply that other molecular mechanisms may also merit consideration.

Another factor may be that treatments of hypoxia have frequently resulted in failure in the past. For increasing tissue oxygenation, the focus is usually on the breathing of enriched oxygen gases or the use of 'blood substitutes'. The latter are compounds such as modified hemoglobins or fluorocarbons, which, like breathing 100% oxygen, increase the concentration of oxygen in the plasma. Fick's Law [2] states that increasing the oxygen concentration can increase its diffusion through plasma, as would decreasing the diffusion distance or increasing the diffusion coefficient (such as with TSC).

As is well known, there have been failed clinical trials using blood substitutes. Thus, an obvious question may be why TSC should work when those compounds do not, since the uses of both enriched oxygen and blood substitutes have also shown beneficial results in preclinical studies. Perhaps the answer to that question may lie in examining the results of an Israeli group that investigated using 100% oxygen for resuscitation from hemorrhagic shock, and comparing their results to those obtained with TSC.

In the first Israeli study [31], they administered 100% oxygen to rats soon after the hemorrhage ended. As in the results seen when using TSC, they found that blood pressure increased, to the same extent and over the same time course as it does with TSC, and survival was also increased. However, when they waited 20 min after the hemorrhage before administering oxygen [32], they found that the blood pressure increased at first for a short time but then decreased at an even faster rate than it did for the controls.

It will be remembered that when TSC was given 20 min after the hemorrhage, it took repeated bolus injections in order to see a sustained increase in the blood pressure [9]. In another study in which the blood pressure was kept at a low level for 30 min before treatment [10], it was also found that a single bolus of TSC gave only a transient rise in blood pressure. However, once again, repeated boluses resulted in a sustained increase in blood pressure.

In summary, in a rat hemorrhagic shock model, a single bolus injection of TSC or the use of 100% oxygen worked equally well if the animals were treated immediately. However, if either treatment were delayed for 20 min (meaning that the hypoxia had persisted for a longer period), neither a single injection of TSC nor the use of 100% oxygen caused more than a transient rise in blood pressure. Since repeated injections of TSC did result in a sustained blood pressure rise, this might suggest that the effects of increasing oxygen

diffusion with TSC or by giving oxygen (or perhaps using a blood substitute) depends on the acid state of the blood (which worsens over time as hypoxia persists). It is possible that changes in acidity could decrease the hydrogen bonding of the water molecules to each other and thereby reduce the diffusivity of oxygen. Perhaps repeated 'assaults' on the water structure with TSC finally overcome the effect of the lower pH. Increasing the oxygen concentration of the plasma does not alter the water structure; thus, this could suggest that neither enriched oxygen nor blood substitutes will be beneficial when the blood pH has been lowered. These, of course, are speculations that need further experimentation to prove or disprove.

Another factor favoring the use of TSC for hypoxia/ischemia is that it increases the diffusion of all reactants to the tissue mitochondria. Thus, the overall metabolism remains in balance. On the other hand, use of 100% oxygen or blood substitutes increases the diffusion of oxygen but not of other reactants such as glucose. This might result in a metabolic

'stress', in that there might not be enough glucose to react with the additional oxygen.

TSC represents a new approach to treating hypoxic situations, and, as such, will require more studies to completely understand and verify its promise. Diffusion Pharmaceuticals believes that clinical proof of the action and efficacy of TSC offers the best opportunity to widen its acceptance and use.

Acknowledgements

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Declaration of interest

JL Gainer is the Chief Scientific Consultant for Diffusion Pharmaceuticals LLC.

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