



Hypersaline Infusion Protocol through the Portal Vein may Focus Electroporation on Tumor Tissue, but is it really Safe? Preliminary Results

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Abstract

Introduction: Irreversible Electroporation (IRE) is highly dependent on the electrical conductivity of the tissue and the high conductivity of tumor tissue, which leads to a lower field than in the surrounding healthy tissue. Hypersaline Infusion (HI) through the portal vein focuses IRE on scattered liver tumors, by creating a differential conductivity between the different types of tissue.

The aim of this study is to determine the effects of the HI protocol on the hepatic and histological biochemical results.

Methods: Ten male Sprague Dawley rats were used for HI protocol. Blood samples were collected at pre-, immediately post-, 24-hrs, 72-hrs, 1-week and 3-weeks post-HI. All the animals were sacrificed after one-month follow-up in order to collect histological samples.

Results: The mortality rate in this procedure reached 30% (3/10). Only the pH and transaminases at 24-hrs were significantly and directly linked to mortality ($p=0.036$ and $p=0.004$, respectively). The three non-surviving animals had a four-time higher AST level at 24-hrs. Natremia normalized at 24-hrs post-HI. Statistically significant differences were found in hepatic necrosis between the non-surviving ($n=3$) and surviving rats ($n=7$) (30.67 ± 10.97 vs. $2.86 \pm 7.56\%$ respectively, $p=0.01$).

Discussion: HI through the portal system involves a significant risk of possibly lethal cytolysis and acidosis. Therefore, compensatory measures and a reduced saline overload are warranted to improve the survival rates.

Introduction

Electroporation (EP) has become a popular non-thermal ablation technique because of its many applications, ranging from the cancer treatment field (irreversible electroporation-IRE) [1-4] to *in vivo* and *in vitro* gene transfection or electrochemotherapy (reversible electroporation-RE) [5,6]. EP applies a high-pulsed electric field to the targeted biological cells, which increases cell membrane permeability and leads to either cell apoptosis or necrosis [4,7]. IRE has been reported to have two main advantages over other thermal ablation techniques such as radiofrequency or microwave: 1) the results are not influenced by the so-called *heat sink effect* in the vicinity of large vessels, which may help to avoid tumour recurrences, and 2) the extracellular matrix of the supportive connective tissue and adjacent vital structures are spared during ablation [8]. However, IRE is also highly dependent on tissue electrical conductivity and as tumor tissue is normally highly conductive, this leads to a lower electrical field than in the surrounding healthy tissue [9].

We recently published a preliminary study in which the conductivity of the healthy tissue was selectively increased by means of hypersaline infusion (HI) through the portal vein. In this study we found that healthy tissue conductivity could be up to 1.4 times higher (1.04 - 1.76 range) than tumor tissue [9]. These different conductivities could be used to focus IRE application on scattered liver tumors. In this regard, Qasrawi et al. [10] highlighted the impact of conductivity heterogeneities

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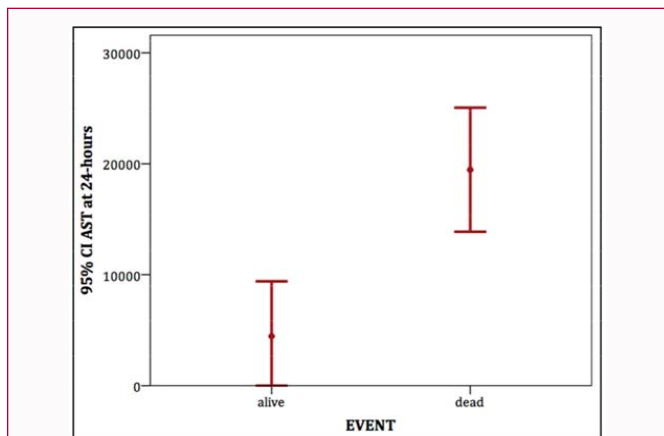


Figure 1: Relationship between AST at 24-hrs with the dead or alive animals, in which AST was four-times higher in dead rats.

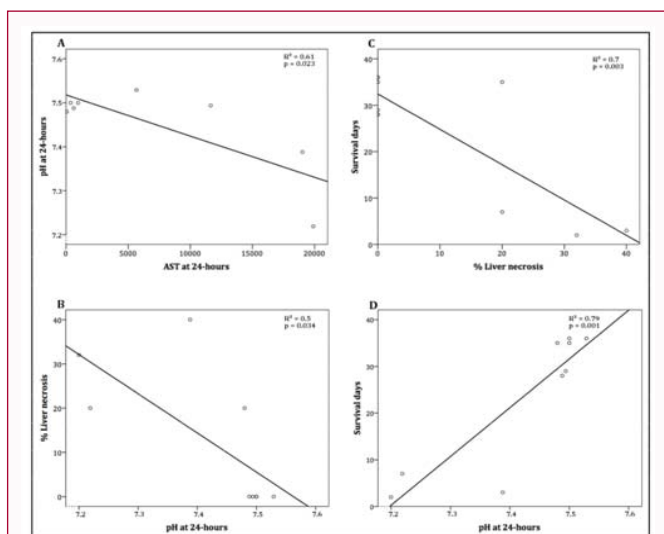


Figure 2: Linear regression underlining the correlation between acidosis at 24-hrs with cytolysis (AST at 24-hrs), hepatic necrosis and survival. A) Represents negative linear relationship between pH at 24-hrs and cytolysis (AST 24-hrs). B) Also shows an inverse relationship between acidosis (pH at 24-hrs) and percentage of necrosis at autopsy. C) Shows the impact of acidosis on the number of days of survival (4 ± 2.65 for dead rats ($n=3$)). D) Reflects the relationship between the percentage of hepatic necrosis of dead rats and days of survival.

and the electric field distribution on treatment outcomes. In fact, other authors have also underlined the importance of the differential electrical conductivity of the liver parenchyma and blood vessels, since vessels have higher conductivity that could have an impact on the distributed electrical field [8,10,11]. It therefore seems possible to modify the baseline electric field around large vessels, especially when the aim is a systemic treatment over an entire area with multiple metastatic lesions. Many studies have been based their therapies on homogenizing the local electrical field; for example, better electroporation outcomes have been obtained in cutaneous tumors using plate electrodes between the treated tissue to increase the surface contact [12] and conductive gels have been used to homogenize the electric field for superficial cutaneous tumors [13]. However, in deeper tumors such as those found in metastatic livers the heterogeneity of the local field has to be taken into account, and not only the shape and position of the electrodes and voltages used [14,15], but also the conductivity of the tissue to be treated.

The authors consider that HI could be a valuable therapeutic tool when used to increase the conductivity of healthy hepatic tissue in order to protect it against tumor tissue (without portal irrigation) due to its high conductivity and the fact that it is easily eliminated by the kidney. In a pioneering study [16] we assumed that the contribution of sodium (Na^+) during HI is exclusively to the extracellular liquid and can determine electrolyte imbalance, such as hyponatraemia [17,18], but we did not assess HI side effects. More importantly, we did not assess the post-HI histological changes over time or the ionic disturbances it could cause.

In this context, the purpose of the present study was to determine the effects of the HI protocol on the hepatic biochemical results, as well as its histological effects on the liver and other organs.

Materials and Methods

The Government of Catalonia's Ethics Committee on Animal Research approved this study on a small animal model to analyze the electrolytic changes after HI (FBP-13-1474P2 procedure, DAAM: 7016). This animal research protocol was conducted following Directives 2010/63/EU of the European Parliament and Council of 22 September 2010, for the protection of animals for experimental and scientific purposes.

Animal model

Ten six-week-old male Sprague Dawley rats (Charles River Laboratories, Kingston, NY, USA) were used to carry out blood tests during and after the HI protocol. All the animals were maintained under standard conditions with the appropriate diet and water *ad libitum*. All the animals were supervised following Morton and Griffiths' guidelines on the recognition of pain, distress and discomfort [19] and sacrificed one month after the HI protocol.

Hypersaline infusion protocol

The same HI protocol was used as previously described in [9]. Intravenous furosemide (loop diuretic, 2 mL/Kg, Seguril Sanofi, Spain) was administered pre and post-HI protocol to compensate the fluid overload. The procedure entails the administration of $\text{D}_2\text{H}_2\text{O}$ (Agua para preparaciones inyectables, Grifols, Spain) and NaCl 20% (Cloruro sódico, 20% Grifols, Spain). The analgesic regimen and antibiotic were administered according to the protocol previously described.

Blood tests

Each animal was given six blood tests during the protocol. The samples pre- and immediately post-HI were obtained through the jugular catheter during HI, and then at 24-hrs, 72-hrs, 1-week and 3-weeks post-HI, blood samples were also collected from the lateral vein tail. The electrolytes (Na^+ and K^+), Hemoglobin (Hb), haematocrit and blood gases (HCO_3^- , BE, pH, pCO_2 , pO_2 and SatO_2) from all the animals were analyzed immediately with an i-STAT[®] device (Abbott Point of Care Inc, Princeton, NJ, USA) at pre, post-HI and at 24-hrs post-HI (0.1mL). Five sixths of the samples (0.5 mL) were kept to analyze liver biochemistry (aspartate aminotransferase-AST, alanine aminotransferase-ALT) and kidney function (creatinine). The 24-hrs to 3-weeks post-HI samples were kept in tubes with an anticoagulant (0.5 mL). The centrifuged serum was carefully removed using a pipette and kept at -4°C for biochemical analysis after the post-surgical follow-up period.

Histopathological samples

Autopsies were performed on the sacrificed or dead animals. Liver,

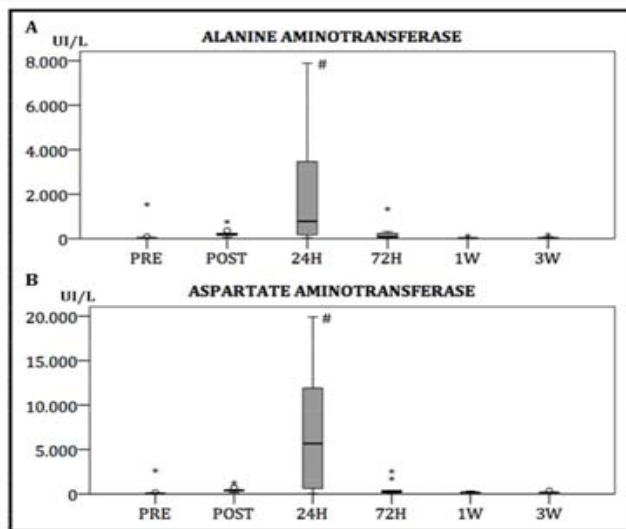


Figure 3: Box plots present ALT and AST pre- and post-HI, 24 and 72-hours post-HI and 1 and 3-weeks post-HI. Severe hepatocytolysis was observed: ALT at 24-hrs was 2286.53 ± 2971.77 vs. 50.71 ± 54.32 U/L at 3-weeks post-HI, $p=0.018$. AST at 24-hrs was 7795.33 ± 8074.62 vs. 159.29 ± 100.64 U/L at 3-weeks post-HI, $p=0.028$. (#) indicates the most important parameters that reached statistical significance.

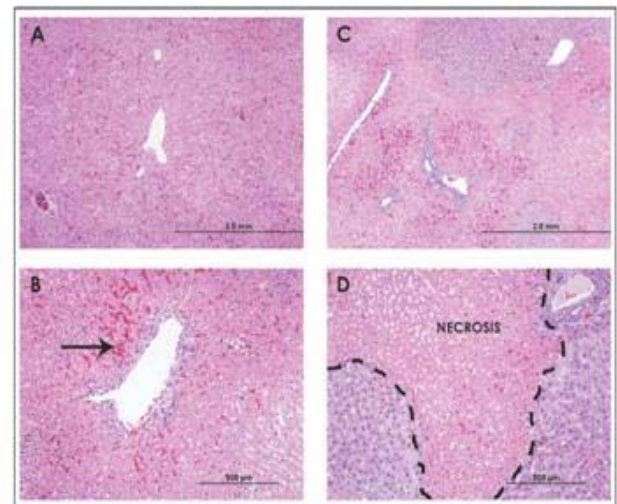


Figure 4: A) and B) represent a normal liver after HI protocol with minimum vascular congestion at 4x and 10x of surviving rats sacrificed at 30 days. Areas of sinusoidal vascular congestion are indicated by (←). C) and D) represent hepatic parenchyma with hepatocytes zonal necrosis at 4x and 10x of dead rat at 48-hrs post-HI (area of hepatic necrosis indicated by dotted line).

spleen, kidneys, lungs and heart were collected, 4% formalin-fixed and paraffin-embedded. Sections of $3 \mu\text{m}$ were cut for haematoxylin-eosin staining. The histological analyses were performed by pathologists with no prior knowledge of the animals' history.

The histological assessment procedures were the following: preservation of parenchyma from different organs (qualitative analysis), percentage of liver vessel congestion (semi-quantitative); quantity of tissue necrosis in liver and spleen (mean percentage of 10 fields chosen at random at 200 x); sinusoidal obstruction syndrome or sinusoidal dilatation in liver (assessed semi-quantitatively according to the Rubbia-Brandt classification [20]).

Statistical analyses

All the statistics were processed by the SPSS statistical software package (SPSS, Version 21, IBM, and Armonk, NY, USA) and expressed as mean \pm standard deviation.

The Mann-Whitney U test was used for non-parametric data to compare blood test results and death/sacrifice events. The Friedman test was used for non-parametric data to analyze differences between the blood test results over time. The Wilcoxon test for pairwise comparisons was carried out on all the analytical parameters. Linear regression was used to correlate acidosis and hypertransaminasemia with hepatic necrosis and survival. A p-value of <0.05 was considered statistically significant.

Results

Cytolysis and acidosis: key elements in the postoperative period

The mortality rate in this procedure reached 30% (3/10). Two of the ten animals were sacrificed at 72-hrs and 1-week post-HI, both due to respiratory difficulties and lethargic behavior. One animal that had shown similar behavior was found dead at 48-hrs post-HI.

From all the analytical results, only pH and transaminases at the 24-hrs were significantly and directly linked to mortality ($p=0.036$

and $p=0.004$, respectively). In fact, the rats with poor evolution had four-time higher AST at 24-hrs (Figure 1), often associated with lower pH at 24-hrs. Furthermore, a negative linear relationship was found between pH at 24-hrs and cytolysis ($R^2=0.61$; $p=0.023$) (Figure 2A and Figure 3) and also in the rate of necrosis at autopsy ($R^2=0.5$; $p=0.034$) (Figure 2B). Not surprisingly, the three dead rats had extended hepatic necrosis and again a negative linear relationship was observed with survival ($R^2=0.7$; $p=0.003$) (Figure 2C). As expected, pH at 24-hrs was also related to survival ($R^2=0.79$; $p=0.001$) (Figure 2D).

Review of other biochemical parameters

Despite the compensation with hyposaline infusion, the mean post-HI natremia (155.4 ± 5.02 mmol/L) was higher than preoperative values ($p=0.005$) but normalized at 24-hrs post-HI (124.63 ± 18.22 mmol/L) and mortality was not related to natremia. On the contrary, post-HI kalemia did not remain stable and no relation to either acidosis or mortality, nor there were significant signs of renal failure (Table1).

Histopathological evaluation

The hepatic parenchyma exhibited a normal architecture in seven of the ten rats after the HI protocol, with polygonal hepatocytes and preserved portal triads. Some dispersed congestion was observed in vessels throughout healthy hepatic tissue, mostly in two-thirds of the parenchyma (Grade 2) [20]. However, the sacrificed or dead animals had wide areas of variously distributed hepatic coagulating necrosis and a sparsely-preserved liver architecture. The differences in hepatic necrosis between the non-surviving ($n=3$) and surviving rats ($n=7$) were statistically significant (30.67 ± 10.97 vs. $2.86 \pm 7.56\%$ respectively), $p=0.01$ (Figure 4).

There was no significant damage of the kidney and lung parenchyma or myocardial tissue.

Discussion

IRE is a promising and relatively new technique that is considered

Table 1: Mean \pm standard deviation of blood test results from 10 Sprague Dawley rats. Blood samples extracted from jugular vein for the pre and post-HI protocol and from the lateral tail vein for the 24-hr and 72-hr post-HI, 1-week and 3-weeks after surgery. Normal values for blood test and venous blood gases [32,33].

	p _r H	p _r CO ₂	p _r O ₂	BE	HCO ₃	TCO ₂	Sat O ₂	Na ⁺	K ⁺	Hct	Hb	ALT	AST	Creat	Normal values
Pre	7.24 \pm 0.05	70.8 \pm 11.82	70.11 \pm 14.33	3.8 \pm 2.14	30.99 \pm 2.41	33.1 \pm 2.66	88.78 \pm 5.16	137 \pm 2.19	4.27 \pm 0.41	41.3 \pm 2.41	14.04 \pm 0.81	187.35 \pm 473.57	330.3 \pm 805.1	0.38 \pm 0.29	PH 7.38 \pm 0.12
Post	7.18 \pm 0.09	62.36 \pm 14.19	84.1 \pm 31.31	5.4 \pm 2.42	23.05 \pm 2.15	25.1 \pm 2.59	90.5 \pm 5.14	155.4 \pm 4.76	4.35 \pm 0.65	27.5 \pm 2.8	9.36 \pm 0.94	233.84 \pm 202.83	480.7 \pm 303.67	0.31 \pm 0.05	PCO ₂ 37.17 \pm 13.93mm PO ₂ 43.42 \pm 15.94mm BE -1.29 \pm 2.87 m Eq/L
24-hours	7.42 \pm 0.12	35.03 \pm 11.78	94.78 \pm 67.39	-1 \pm 8.51	23.37 \pm 7.21	24.56 \pm 7.6	85 \pm 16.1	124.75 \pm 16.86	7.4 \pm 1.92	29 \pm 5.1	9.84 \pm 1.73	2286.53 \pm 2971.77	7795.33 \pm 8074.62	0.53 \pm 0.25	HCO ₃ 24.53 \pm 3.33 m Eq/L Sat O ₂ 70.6 \pm 17.33%
72-hours									6.84 \pm 1.2			256.38 \pm 417.27	634.57 \pm 875.49	0.49 \pm 0.27	Na ⁺ 150 \pm 3.4 mEq/L K ⁺ 7 \pm 0.65 mEq/L
1-Week												36.89 \pm 35.79	156.38 \pm 86.97	0.87 \pm 1.27	Hct 41.6 \pm 5%
3-weeks												50.71 \pm 54.32	159.29 \pm 100.64	0.44 \pm 0.19	Hb 14.2 \pm 0.5 g/dl ALT 49 \pm 24.1 U/L AST 95 \pm 31.7 U/L Creat 2.5 \pm 1.25 mg/dL

to preserve the tissue scaffold, which would be advantageous when treating tumors next to vessels or biliary ducts [21,22], although it does not preferentially ablate tumor over healthy tissue. In fact, due to the higher conductivity of tumor tissue IRE may ablate healthier than tumor tissue [8,23-25]. This is especially true when a sandwich electrode position is applied to the scattered tumor tissue deep inside healthy liver tissue [13,26,27]. A possible approach to overcome this shortcoming would be to increase the conductivity of the healthy tissue by means of hyperconductive solutions such as hypersaline serum, as has already been demonstrated [9].

The aim of the present study was to determine the safety of using a relatively large amount of a highly concentrated saline solution (20%), which has been shown to be beneficial for focusing tumor tissue over healthy tissue during IRE [9,28].

After directly infusing a large amount of saline solution into the portal system, a well-established cytolysis (sometimes over 15,000 u/L) was observed at 24-hrs, even when correct systemic compensation of natremia was achieved (as shown by the Na⁺ levels after the procedure). This acute liver damage probably led to deficient lactate clearance, which could account for the drop in pH at 24-hrs, especially in the non-survivors (all between 48-hrs and 1-week). In fact, a good correlation was found between cytolysis, acidosis and survival, and almost 80% of the survival rate can be directly related to acidosis, suggesting that a specific acidosis treatment at 24-hrs (which was absent) could have avoided mortality. The liver is known to play an important role in regulating the acid-base and the metabolism of organic acid anions like lactate and certain amino acids [29,30].

Surprisingly, the rate of hepatic necrosis, which was closely related to survival, was unexpectedly low (0% in 60% of the cases) compared to such hypertransaminasemia at 24-hrs, even in the dead animals (which was always under 40%) and by itself did not account for the mortality. This phenomenon could only be understood by the rapid hepatocytes regeneration of the surviving rats, which was confirmed at the necropsy by the low necrosis rate, suggesting that hepatic insufficiency could have been the main factor associated with mortality.

Several limitations of the study as regards the infusion protocol and the methodology used should be pointed out. The acidosis and mortality results indicate that lactate should be carefully monitored and counterbalanced, even beyond 24-hrs of the postoperative. It is also clear that the actual infusion protocol, which advantageously focuses the IRE application onto the tumor, can sometimes lead to unavoidable cytolysis. For this reason, our group is now working on a new protocol with a lower Na⁺ overload into the liver and with

intrahepatic HI compensation by infusing deionised serum into the hepatic artery. This new approach should improve the differential conductivity between healthy and tumor tissue, given the preferential arterial perfusion of liver tumors [31].

In conclusion, as HI in the portal system leads to a significant risk of possibly lethal cytolysis and acidosis, compensatory measures and a reduced saline overload are warranted to improve the survival rates.

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