Antiproliferative and Apoptotic Effects of Lidocaine on Human Hepatocarcinoma Cells. A preliminary study

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INTRODUCTION

In the last years, numerous studies have focused on the effects of lidocaine infusion on postoperative pain, recovery and short term outcome [1-5]. It has been demonstrated that lidocaine infusion accelerates bowel movements, reduces postoperative pain and opioid needs and length of hospital stay [2-4]. Anti-inflammatory, analgesic and anti-hyperalgesic effects have been implicated in lidocaine’s clinical effects via different mechanisms including sodium channel blockade [6] and inhibition of G protein-coupled receptor [7].

More recently, a few in vitro and in vivo studies have demonstrated that lidocaine, as well as other local anesthetics, has antiproliferative, apoptotic and cytotoxic effects in cancer cells [8, 9]. Taking into consideration that many of the in vitro studies on cell cultures have used high concentrations of lidocaine in certain tumor cells (e.g. breast), we aimed to investigate if lidocaine in clinical concentrations has the same effects on hepatocarcinoma cells. Moreover, taking into consideration that the liver can be infiltrated during different percutaneous or/surgical maneuvers, by finding such properties for lidocaine will raise the hypothesis of the possibility of reducing the tumor size in the liver or the risk of...
recurrences after hepatic surgery by lidocaine infiltration or perioperative intravenous infusion.

To the best of our knowledge, this is the first study to investigate the antiproliferative effects of lidocaine on human hepatocarcinoma cells.

**MATERIAL AND METHODS**

**Cell culture protocols**

We investigated lidocaine effects by using two cell lines: one hepatocellular carcinoma cell line (HepG2) and one normal liver fibroblast cell line (LX2) (cell lines were kindly gifted by Professor Gianluigi Gianelli, from the University of Bari, Italy). We tested the effects of lidocaine on both a cancer and a normal liver cell line as we wanted to assess the potential deleterious effects on the normal liver cells and to compare them with those on hepatocarcinoma cells.

HepG2 and LX2 cells were cultivated in MEM (Minimum Essential Medium Eagle) (Sigma Aldrich), supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 µg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). LX2 cells were cultivated in DMEM high glucose (Dulbecco's Modified Eagles Media) (Sigma Aldrich) supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA) and 2mM L-Glutamine (Sigma Aldrich). Cells were grown in a humidified 5% CO₂ – air atmosphere at 37ºC and were passaged with 0.05% trypsin-EDTA every 2-3 days. Cells were detached, centrifuged and then counted using trypan-blue to assess cell number/mL and the cells viability.

**Cell proliferation assay**

The antiproliferative and cytotoxic effects of lidocaine in human tumor and normal cells were assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Monolayer cell cultures were cultivated at sub-confluence before being washed twice with a phosphate buffer solution (PBS) and detached with 0.05% trypsin-EDTA. Cells were then re-suspended in culture medium with 10% FBS (Fetal bovine serum), counted, and plated in 200 μL media at 37ºC and were passaged twice with a phosphate buffer. Monolayer cell cultures were cultivated at sub-confluence before being washed twice with a phosphate buffer solution (PBS) and detached with 0.05% trypsin-EDTA. Cells were then re-suspended in culture medium with 10% FBS (Fetal bovine serum) and counted. The percentages of viability of HEP G2 cell line exposed to lidocaine were significantly lower than those in the control group (p<0.05). Cell proliferations of the group for 24 and 72 hours were significantly decreased, compared with the control group (p<0.05). Cell proliferations of the group for 24 and 72 hours were significantly decreased, compared with the control group (p<0.05), while at 48 h these results were not significant (Fig. 2).

**Intra-group analysis** is shown in Fig. 3. As can be seen in Fig. 3A, for HepG2 cells, the effects are not significant at 0.5 µM lidocaine (p>0.05) at every time interval as compared to 3 µM, where effects are highly significant (p<0.001) (Fig. 3B).

The same results were registered for LX2 cells (Fig. 4A). In case of LX2 cells, the results were not significant for 0.5 µM lidocaine (p>0.05) at every time interval as compared to 3 µM lidocaine where results were highly significant (**p <0.01, ***p <0.001) (Fig. 4B).

Areas under curve for every time interval and lidocaine concentration in both cell lines were compared to evaluate the antiproliferative effects in both cancer and normal liver cells (Table 1). Lidocaine antiproliferative effects were significantly lower in normal liver fibroblasts as compared with hepatocarcinoma cells, apart from 0.5 µM where there were no significant differences (p <0.001).

**Protein synthesis assessment with Western blotting**

Cells were lysed in Laemmli sample buffer (Bio-Rad, Hercules, California, USA) supplemented with a protease inhibitor complete EDTA-free (Roche, France). Protein concentration was measured using BCA Protein Assay kit (Pierce, Rockford, Massachusetts, USA). Cell lysates (50 µg) were electrophoresed on 10–20% polyacrylamide gels (Bio-Rad) and transferred to ImmobilonPSQ membranes (Millipore, Bedford, Massachusetts, USA). The membranes were blocked with TBS containing 5% skim milk and 0.1% Tween-20, and then incubated with the primary antibody. Antibody to p53 was purchased from Abcam (Cambridge, United Kingdom). The membranes were incubated after washed with HRP-conjugated goat anti-rabbit IgG (Calbiochem, Gibbstown, New Jersey, USA) and analyzed using enhanced chemiluminescence-plus reagent (GE Healthcare, Buckinghamshire, United Kingdom).

**Statistical analysis**

The statistical analysis was performed using R (R Development core team, USA) and GraphPad Prism 5.0 (GraphPad Software INC, CA, USA). The obtained data was first examined for normality of distribution using the Shapiro-Wilk test. The distribution of all the obtained data was Gaussian; thus they were analyzed using the t-test. For each concentration, we calculated the AUC (area under the curve) using the trapezoidal method. In order to evaluate the AUCs trend regarding the different concentrations of lidocaine and type of cell line, we used the two-way ANOVA test. A p <0.05 was considered significant.

**RESULTS**

**Cells proliferation assay**

Proliferation of HepG2 and LX2 cells exposed to different concentrations of lidocaine was determined at 24, 48 and 72 hours. The percentages of viability of HEP G2 cell line exposed to lidocaine were significantly lower than those in the control group (p<0.05). Compared to control, cell proliferation was significantly decreased in lidocaine exposed cells for every time interval (p<0.05) (Fig. 1). As can be seen in Fig. 1, the decrease in cells proliferation is time-concentration dependent.

Similarly, the percentages of viability for LX2 cell lines at 24, 48 and 72 hours were significantly lower than those in the control group (p<0.05). Cell proliferations of the group for 24 and 72 hours were significantly decreased, compared with the control group (p<0.05), while at 48 h these results were not significant (Fig. 2).

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**Protein p53 degradation**

To investigate a possible mechanism for lidocaine antiproliferative effects in cancer cells, we assessed the
degradation of the p53 protein in both types of cells (HepG2 and LX2) treated with lidocaine, as compared with cells cultured without lidocaine (Fig. 5).

The total amount of p53 protein degraded in HepG2 cancer cells exposed to 1.75 µM lidocaine was significantly higher as compared with the amount degraded when LX2 cells were exposed to the same concentration of lidocaine (p = 0.0241) (Fig. 5).

The quantitative analysis for protein degradation is shown in Fig. 6: there was a significant difference between the HepG2 cells treated with lidocaine and without (p = 0.0007).

Fig. 1. Evaluation of the effect of various concentrations of lidocaine on HepG2 human hepatocarcinoma cells at 24, 48 and 72 h using MTT assay.

Fig. 2. Evaluation of various concentrations of lidocaine on LX2 normal liver fibroblasts at 24, 48 and 72 h using MTT assay. *p<0.05, **p<0.01, ***p <0.001.
Fig. 3. A and B. Evaluation of the effect of 0.5 µM and 3 µM concentrations of lidocaine on HepG2 human hepatocarcinoma cells at 24, 48 and 72 h (**p <0.001).

Fig. 4. A and B. Evaluation of the effect of 0.5 µM and 3 µM concentrations of lidocaine on LX2 normal liver fibroblasts at 24, 48 and 72 h (**p <0.01, ***p <0.001).

Table I. Area under curve (AUC) for the antiproliferative effects of different lidocaine concentrations in HepG2 and LX2

<table>
<thead>
<tr>
<th>Lidocaine Concentration (µM)</th>
<th>AUC* HepG2</th>
<th>AUC* LX2</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>4944.30 ± 216.57</td>
<td>4944.29 ± 340.00</td>
</tr>
<tr>
<td>0.5</td>
<td>4284.21 ± 134.29</td>
<td>4862.22 ± 548.85</td>
</tr>
<tr>
<td>0.75</td>
<td>4092.45 ± 42.53</td>
<td>4922.04 ± 554.20</td>
</tr>
<tr>
<td>1</td>
<td>3849.62 ± 81.34</td>
<td>4699.32 ± 391.31</td>
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<tr>
<td>1.25</td>
<td>3648.34 ± 148.31</td>
<td>4654.69 ± 205.13</td>
</tr>
<tr>
<td>1.5</td>
<td>3524.72 ± 151.04</td>
<td>4813.88 ± 187.50</td>
</tr>
<tr>
<td>1.75</td>
<td>3266.68 ± 127.82</td>
<td>4368.93 ± 206.39</td>
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<tr>
<td>2</td>
<td>2936.85 ± 73.05</td>
<td>4304.49 ± 308.85</td>
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<tr>
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<td>2393.00 ± 54.48</td>
<td>4214.26 ± 240.79</td>
</tr>
<tr>
<td>3</td>
<td>1877.87 ± 50.07</td>
<td>3483.88 ± 184.09</td>
</tr>
</tbody>
</table>

* p < 0.001

**DISCUSSION**

The findings of our study suggest that, in clinically relevant concentrations, lidocaine has significant antiproliferative effects on human hepatocarcinoma cells. Previous studies reported antiproliferative, apoptotic and cytotoxic effects for different plasma concentrations of lidocaine on various types of tumor cells [9-12]. Thus, in a concentration used for infiltration during surgical interventions (5-20 mM), lidocaine inhibited the invasive ability of different human cancer (HT1080, HOS, and RPMI-7951) cells by inhibiting the shedding of HB-EGF from the cell surface and by modulating intracellular Ca²⁺ concentration [9]. Although not accepted by all authors [9], lidocaine's sodium channel blocking activity may also contribute to the anti-invasive effect [13-15]. The voltage gate sodium channels (VGSC) are strongly expressed in cancer cells promoting motility and invasion of the cells [15].

On the other hand, local anesthetics cause increased permeability of the mitochondrial membrane, collapse of the mitochondrial membrane potential, and decrease in adenosine triphosphate production by either uncoupling of oxidative phosphorylation or inhibition of complex I of the mitochondrial respiratory chain [15]. In addition to their effect on mitochondrial bioenergetics, lidocaine, bupivacaine, and tetracaine were shown to cause DNA fragmentation and membrane blebbing, activate caspases, and release cytochrome
Sakaguchi et al. [12] investigated the effects of lidocaine (40–4000 µM) on the proliferation and epidermal growth factor (EGF)-stimulated autophosphorylation of the EGFR in human tongue cancer cells CAL27, which has a high level of EGFR expression. They found that a clinical concentration of lidocaine (400 µM) suppressed both serum-induced and EGF-induced proliferation of CAL27 cells and inhibited EGF-stimulated tyrosine kinase activity of EGFR without cytotoxicity. An increased concentration of lidocaine, of 4000 µM, expressed both cytotoxicity and antiproliferative effects. The authors suggest that the inhibition of EGF-stimulated EGFR activity is one of the mechanisms of the antiproliferative effect of lidocaine on CAL27 cells and lidocaine administered topically within the oral cavity for cancer pain relief may suppress the proliferation of human tongue cancer cells.

Similar to other studies [16], our results showed that antiproliferative effects of lidocaine were time- and dose-dependent. As compared with other studies, we used nine different low (as compared with other studies) concentrations of 0.5-3 µM lidocaine for exposure of HepG2 cells for 72h. To our knowledge, this is the first study that evaluated antiproliferative effects of nine different clinically relevant concentrations of lidocaine on human hepatocarcinoma cells.

Our results showed that, in concentrations of more than 0.5 µM, lidocaine has significant antiproliferative effects. For concentrations of 0.5 µM these effects were not significant. It is interesting that in the same low concentration, lidocaine seems not to inhibit „protective immunity”. Studies published so far found that, in low concentrations such as those in patients with continuous infusion, lidocaine enhanced the in vitro function of NK cells by the release of lytic granules [17]. In these concentrations lidocaine has also antiinflammatory effects by inhibiting PMN accumulation and pro-inflammatory interleukin release, but these effects favor an improved postoperative rehabilitation (pain score, resumption of bowel function, etc) [18]. Randomized clinical trials focused on these effects (e.g. NK cells) are currently enrolling patients.

In our study, we further wanted to investigate a possible mechanism for lidocaine antiproliferative effects in cancer cells by investigating lidocaine effects on p53 expression in both hepatocarcinoma cells and normal fibroblasts.

Protein p53 plays an important role in mediating cell response to various stress factors, mainly by suppressing a number of genes implicated in cell cycle arrest, apoptosis, senescence, DNA repair and angiogenesis [19-21] and is the most important tumor suppressor protein [22]. Wild-type p53 is a potent inducer of apoptosis when expressed in cancer cells; however, in these cells, most often mutants p53 are expressed [22] in which loss of wild-type function may be present.

In our study, the expression level of p53 was significantly reduced in hepatocarcinoma cells exposed to lidocaine. A possible hypothesis for our results may be that lidocaine decreased the expression of mutants p53 that were not detected by our method and may have been increased in the cancer cell line.

Thus, one of the limitations of our study is that our methods did not detect the possible mutant p53 that may have been otherwise increased in hepatocarcinoma cells. Another limitation is the low impact in clinical practice of these findings. Our results on the in vitro antiproliferative effects of lidocaine in concentrations above 0.5 µM will have to be confirmed by future animal models and clinical studies.

Taking into consideration that the clinical impact of in vitro cell cultures studies is low, further clinical studies are required to quantify the effect of lidocaine infiltration/infusion on the incidence of recurrences and survival in patients with hepatocarcinoma and to determine if lidocaine has an inhibitory effect on wild-type or mutant p53.

**CONCLUSION**

Our study showed that, in different clinically effective concentrations, lidocaine has antiproliferative effects in hepatic carcinoma cells. These effects can be at least partially explained by lidocaine’s influence on the expression level of protein p53. These premises lead to potential additional useful clinical effects of lidocaine infiltration of the hepatic parenchyma in patients with hepatocarcinoma undergoing surgery or minimally invasive (percutaneous) maneuvers. The same effect may be produced by perioperative i.v. lidocaine infusion in patients with hepatocarcinoma.
Conflicts of interest. Nothing to declare.

Authors’ contribution. D.I. and C.T.: design of the study and major contribution to draft manuscript; A.J., T.T., L.B.N. and C.T.: cell cultures, data interpretation and manuscript revision; S.V.: statistical analysis and interpretation, manuscript revision. All authors approved the final version of the manuscript.

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REFERENCES