Abstract

**Aim:** The aims of this study are the development of a contrast enhanced ultrasonography (CEUS) protocol for rats’ evaluation and the assessment of the potential benefits of CEUS in Walker 256 tumor rats. **Material and method:** In the study were used 36 albino Wistar rats grafted subcutaneously with Walker 256 tumor. The implementation of the ultrasound (US) guided injection technique (30 subjects – group A) was performed between 4 to 8 weeks after implantation. The contrast agent (CA) - Sono Vue (Bracco) – was administered either into the lateral vein of the tail or directly into the heart under US guidance. The US validation, focusing on CEUS (6 subjects – group B) was realized at 4 to 6 weeks after implantation. The US procedures aimed to obtain morphological (2D), vascular (color Doppler and pulsed Doppler) and angiospecific functional data (CEUS). The Vevo 2100 equipment was used for US and Time Intensity Curves (TIC) were analyzed with Sonoliver (TomTec). The tumor specimens which were resected after the last study underwent a pathology exam. **Results:** A number of 23 successfully CEUS explorations were performed in 17 subjects (11 in group A and 6 in group B). Nineteen rats could not be evaluated (in 8 cases the tumor was not viable; 4 subjects died during CA administration; in 4 cases the administration line could not be obtained). In group B, CEUS was performed in 6 subjects at 4 weeks after implantation and in 5 subjects at 6 weeks. The statistical analysis of the TIC parameters identified significant differences between the Time to Peak, mean Transit Time and Rise Time parameters of the muscles and those of the tumor. **Conclusions:** CEUS was easily implemented on the studied tumor model and is adequate for the evaluation of tumor vascularity. US guided intracardiac administration of the CA is an easy and reproducible procedure. If the examination is performed at defined time intervals it detects the alterations within the tumor circulatory bed. **Keywords:** ultrasonography, contrast agent, rat, Walker 256 tumor

Introduction

The therapy of oncological entities has been under constant development in the last decades. Judah Folkman realized in 1971 one of the most important discoveries in this area, by developing the hypothesis of the fundamental role of angiogenesis in tumor development [1]. Currently, angiogenesis is recognized as a „sine qua non” event in the development and dissemination of tumors [2,3], while a new class of antitumor agents, antiangiogenic drugs, is under continuous development [4]. Present evaluation criteria for oncological treatment response, are mainly focused on the morphological assessment of tumors and thus, are subjected to a limited evaluation capacity [5,6]. The RECIST system, for example, takes into consideration only the size of the
tumor [7]. Integrated tumor assessment criteria, based on the morphological changes, must be completed with other explorations, while there is a need to develop new surrogate markers of the therapy response [8]. Recent improvements in the radio-imaging techniques, including contrast enhanced ultrasonography (CEUS), could gain a special place for the evaluation of treatment response.

Clinical trials have demonstrated that tumor volume is not significantly reduced in the initial phases of the antiangiogenic therapies [9]. This evolutive feature is especially important, both because of the imaging procedures involved in the quantification of the therapy response and because of the disease prognosis and conduct towards the patient. The non-invasive evaluation of the tumor circulation is even more important.

Doppler ultrasonography is not sensitive enough to detect subtle vascular changes in the early phases of the therapy [10]. CEUS represents a promising non-invasive method in the early evaluation of the therapy response since it makes it possible to visualize and quantify tumor vascularity “in vivo” [11-14]. Contrast agents used in ultrasonography consist of a gas enveloped in a protein or carbohydrate capsules which result in 10 μm microbubbles. CEUS is characterized by high spatial resolution, is performed in real-time and is easily applied both on human patients and animal subjects. There are several types of contrast agents (CA) used in ultrasonography (ex.: SonoVue, Levovist, Sonazoid). The one that was approved for clinical use in the EU is SonoVue (Bracco Spa, Milan) [15].

Animal models play an important role in the development of the new oncological therapies and in the validation of the new imaging techniques proposed for the evaluation of the therapeutic response. Therefore, selecting an adequate experimental model is extremely important [16].

The rat Walker 256 tumor bears the name of its discoverer, Prof. G Walker who identified it in 1928, as a spontaneous breast tumor in the pregnant albino rat [17]. Since then the tumor has been used in many model experiments on rats [18,19].

Ultrasound studies on rats bearing Walker 256 carcinoma have mainly focused on the detection of occult metastases [20] and on the treatment of the tumor using ultrasound cavitation techniques [21] or ultrasound thermotherapy [22].

The aims of our study were a) to develop a protocol for the CEUS examination of the albino Wistar rats grafted with Walker 256 tumor; b) to assess the potential benefits derived from applying the CEUS technique to the Walker 256 rats experimental tumor model.

### Material and method

**Animals and tumor model.** The study involved 36 albino Wistar male rats carrying Walker 256 carcinosarcoma implants (weight 180-200 g, age 3 months). The subjects were randomly divided into two groups, group A (n=30 subjects) and group B (n=6 subjects). Group A was assigned to establish the protocol needed to be used for the CEUS examination, while group B was used for the assessment of CEUS potential benefits regarding tumor vascular characterization. All the animals were obtained from the Biobase of the “Iuliu Hatieganu” University of Medicine and Pharmacy, Cluj-Napoca. The animals were cared for in the Biobase of the Physiology Department of the same University. They were isolated for 10 days prior to their introduction in the study for acclimatization. The animals received a standard diet and their access to water was not restricted. All the experiments were conducted in agreement with the protocols and recommendations of the “Iuliu Hatieganu” University of Medicine and Pharmacy, Cluj-Napoca, Ethics Committee.

The rats underwent intramuscular anesthesia with Ketamine (80mg/kg) and Xylazine (8 mg/kg). Tumor implantation was achieved by depositing small fragments of the Walker 256 tumor subcutaneously, on the right thigh of the rats. Group A was the first implanted group, followed by Group B, 8 weeks later. For each Group, day 0 is referred as the day in which the tumor implantation was carried out.

**Ultrasoundographic contrast-agent administration.** Before any catheter placement or ultrasound examination, the animals underwent anesthesia according to the previously mentioned method. The contrast agent was administered using single line 26 G catheters. For the first method the animal was placed in ventral decubitus and the catheter was introduced in the lateral vein of the tail. The precision of the puncture was verified by blood aspiration [23]. The second method consisted in placing the animal in left lateral decubitus, with the transducer applied on a previously shaved area of the thorax (fig 1), while the catheter was inserted into the heart, through an intercostal approach, under US monitoring. The procedure was considered successful if the tip of the catheter was visualized inside of the heart and if blood was aspirated (fig 2). The two methods were applied successively in each rat.

**Ultrasoundographic protocol.** All the ultrasonographic evaluations were performed by a single experimented examiner (M.S.). For this study was used a high performance ultrasonographic equipment, especially designed for the examination of small experimental animals (Visual Sonics Vevo 2100, 21 MHz central frequency...
transducer). The examination protocol consisted in a B mode, Color Doppler (CFM) (PRF=1.5 kHz, central frequency = 16MHz) and CEUS examination (Power= 4%, frequency = 18MHz). Tumor morphometry was evaluated (echogenicity and structure of the parenchyma; tumor volume) as well as tumor vascularity (the presence of a Doppler signal evaluated with CFM; the circulatory pattern, central, peripheral or mixed; the penetration of the CA into the region of interest (ROI); the direction of the CA penetration into ROI and its homogenicity). The tumor volume was calculated based on the following formula: 
\[ V = \text{length} \times \text{width} \times \text{height} \times 0.52 \] and was expressed in mm³ (fig 3).

The animals in group A were examined starting at 4 weeks after grafting and weekly until 8 weeks after grafting, while the subjects in group B were examined after 4 and 6 weeks post-grafting.

For the initial confirmation of the tumor, a B mode ultrasound (grey scale) with the hand held transducer was performed. After a viable CA administration line was established (tail vein or intracardiac), the subject was positioned in a lateral left decubitus and the transducer was stabilized with the help of a steady arm. The scanning plane included the maximum tumor diameter and the muscle tissue of the thigh which was underneath. To avoid transmission of the pressure upon the tissue, a layer of 0.5 cm of ultrasonographic gel was applied (fig 4).

The CA (SonoVue) was prepared in agreement with the specifications of the producer. A volume of 0.5 ml was administered in bolus, followed by 0.5 ml of isotonic NaCl solution. A dual mode examination (real-time B-mode and CEUS) was continuously recorded for 3 minutes. For both administration methods (tail vein and intracardiac) the examination was considered successful only if the CA was
visible on the US image. The recorded sequences were processed for Time-Intensity Curve (TIC) analysis using the SonoLiver software (TomTec, Bracco, Italia; http://www.tomtec.cn/end_users/radiology_gi.html).

The ROI were selected within the tumor parenchyma and the adjacent muscle tissue (considered as control tissue). Within the tumor parenchyma, the ROI overlapped the entire tumor parenchyma (Tumor) as well the low uptake (Region 1) and high uptake (Region 2) areas. The Time Intensity Curves registered inside the tumor were compared with the ones registered within the muscles (reference tissue). The parameters obtained as a result of the TIC analysis were Time to Peak (TTP), Rise Time (RT), Mean Transit Time (mTT) and Maximum Intensity (IMAX).

The results of the US and CEUS exams were compared with the pathology slices obtained after the animal was euthanized.

**Pathology studies.** After the last CEUS study (week 8 for Group A and week 6 for Group B) all the animals underwent euthanasia. The tumor was removed and cut in a plane which corresponded with the examination plane. The specimens that were obtained were treated in formaldehyde, included in paraffin, sliced and stained with hematoxylin-eosin. The macroscopic specimens were photographed for comparison with the CEUS images.

The statistical analysis was performed using the GraphPad Prism soft. The statistical test which was applied was “Wilcoxon signed rank test” (p=0.05).

**Results**

**Group A.** After the first ultrasound, 9 of the 30 subjects were excluded from the study because the tumor did not graft. The other 21 subjects which remained in the study were examined using B mode, CFM and Pulsed Doppler ultrasonography (fig 5, fig 6).

The catheterization of the tail vein and the consecutive CEUS exploration were successfully performed in one subject. During these maneuvers 2 subjects were lost most likely due to gas embolism and adverse reactions to the anesthetic.

Four rats were lost during the intracardiac placement of the catheter due to the instillation of the CA and of the saline solution into the pleural cavity and pericardium (these events were confirmed sonographically). In four cases an accurate placement of the catheter was not succeeded (fig 7).

The intracardiac catheter was placed successfully and the CEUS examination performed in 11 subjects (this includes the subject with previous successful tail vein administration and CEUS exploration who also had a successful intracardiac administration and CEUS exam).
There were physiological characteristics of the rats, like increased coagulability and accelerated heart rate, which made the explorations more difficult to conduct. These factors prompted the obstruction of the catheter and determined the dislocation of its tip outside of the left ventricle. The images thus obtained were suitable for morphological (2D) and hemodynamic (Doppler, CEUS) interpretations (fig 8, fig 9).

Histological studies of the tumor specimens showed central necrosis, area of dystrophic calcifications and areas of increased vascularity – especially in the periphery. The parallel evaluation of the section plane obtained by CEUS and the histology section plane identified an overlap of the low uptake areas with the necrosis areas and of the high uptake areas with the highly vascular areas (fig 10).

The subjective observations were sustained by the TIC analysis. Highly vascular areas of the tumor showed increased uptake of the CA compared with the subjacent musculature (fig 11).

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**Fig 8.** Dual mode image (B mode and CEUS) of the rat no 18 at 4 weeks after the subcutaneous implantation of the tumor. CEUS image saved at 22 seconds after the i.v. administration of the CA. The presence of the tumor is noticed on the left and the lack of vascular signal on the harmonic image on the right.

**Fig 9.** Dual mode image (B mode and CEUS) of the rat no 18 at 8 weeks after the subcutaneous implantation of the tumor. The CEUS image is acquired using the accumulation function which allows the visualization of the spatial progression of the CA while saving the previous information. The amplitude of the vascular signal is seen, with irregular, tortuous elements. The neoformation blood vessels involve the entire tumor.

**Fig 10.** A: longitudinal section of the tumor in parallel with the CEUS image (accumulation function), in which are showed the necrotic areas inside the tumor (delineated by the dotted line), the area composed of dead and viable cells in equal proportions (red asterisks) and an area with predominantly viable cells (black asterisk); B: a detail of the area marked with red asterisk, where groups of viable cells can be noted (black arrow) as well as areas of intratumoral necrosis (red arrow); C: a highly vascular area (which consists of mainly medium size vessels) from conjunctival and vascular stroma of the tumor; D: a detail of the necrosis area with coagulative necrosis and a tendency of the dead cells towards calcification; E: a detail of the area marked with red asterisks, presenting apoptotic tumor cells (black arrows- apoptotic cells).

**Fig 11.** The TIC analysis of subject no 18 (Group A) at 8 weeks after inoculation. Green ROI placed in the high uptake parenchyma, purple ROI and yellow ROI placed within the low uptake parenchyma. White ROI placed within the muscles of the thigh.
Group B. All 6 subjects from this group underwent a CEUS exam at 4 weeks after implantation, while only 5 were examined at 6 weeks after grafting. All the subjects received intracardiac CA administration. One subject was excluded from the group due to the adverse reactions suffered during anesthesia, reactions that led to the subject’s death during the second examination.

At 4 weeks, the lesions were predominantly hypoechoic compared to the surrounding muscles on the 2D (“grey scale”) examination. The CFM exam identified a vascular signal mainly on the periphery of the tumor, while CEUS showed uptake in periphery of the lesions with centripetal progression (table I).

At 6 weeks after implantation, the lesions evaluated on 2D sonography (grey scale) presented a polymorphic aspect compared with the previous examination and with the adjacent muscles. The CFM exam revealed a mixed vascularity (peripheral and central). CEUS exploration showed a change of the enhancement characteristics in the sense that it was predominantly mixed, peripheral and central (table II).

The statistical analysis of the tumor volume median, determined on the same group of rats, after the 2 intervals, identified a statistically significant difference (p=0.03), suggesting an active, viable tumor (table III) (fig 12).

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Table I. Group B, B mode, CFM and CEUS characteristics at 4 weeks

<table>
<thead>
<tr>
<th>Subject</th>
<th>Echo-</th>
<th>Homoge-</th>
<th>Tumor</th>
<th>CFM</th>
<th>CA uptake</th>
<th>Pattern of CA</th>
<th>Direction of CA</th>
<th>Homogeneity of CA</th>
</tr>
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<tr>
<td></td>
<td>genicity</td>
<td>genity</td>
<td>volume (mm³)</td>
<td>vascularity</td>
<td>circulatory pattern</td>
<td>velocity (no/ultra high/high/low)</td>
<td>uptake</td>
<td>of uptake</td>
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<td>iso</td>
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<td>no</td>
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<tr>
<td>06</td>
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<td>2372.20</td>
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<td>peripheral</td>
<td>centripetal</td>
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Table II. Group B. B mode, CFM and CEUS characteristics at 6 weeks

<table>
<thead>
<tr>
<th>Subject</th>
<th>Echo-</th>
<th>Homoge-</th>
<th>Tumor Volume (mm³)</th>
<th>CFM vascularity pattern (central/peripheral/mixed)</th>
<th>Uptake Velocity (no/ultra high/high/low)</th>
<th>Uptake Pattern (peripheral, central, mixed)</th>
<th>Direction of uptake (centripetal, centrifugal, undetermined)</th>
<th>Uptake homogeneity (homogeneous, inhomogeneous)</th>
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<td>9260.60</td>
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<td>9317.81</td>
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<tr>
<td>06</td>
<td>hyper</td>
<td>no</td>
<td>11160.24</td>
<td>present mixed high</td>
<td>mixed</td>
<td>centripetal</td>
<td>inhomogeneous</td>
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</table>

Table III. Tumor volume median, minimum and maximum in group B

<table>
<thead>
<tr>
<th>Tumor volume (mm³)</th>
<th>4 wks</th>
<th>6 wks</th>
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</thead>
<tbody>
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<td>Minimum</td>
<td>543.0</td>
<td>1941</td>
</tr>
<tr>
<td>Median</td>
<td>2131</td>
<td>9261</td>
</tr>
<tr>
<td>Maximum</td>
<td>3168</td>
<td>11160</td>
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</table>
The analysis of the TIC parameters obtained after the dynamic CEUS exploration within the studied group (at 4 and 6 weeks after implantation), focused on the reference area (Reference), the tumor tissue (Tumor), the low uptake area (Region 1) and the high uptake area (Region 2) is presented in Table IV.

A statistically significant difference between the medians of the TTP of the Tumor and Reference areas was registered, both at 4 weeks and 6 weeks (p=0.03). Statistically significant differences were also observed between the medians of the RT and mTT (p=0.03), for the same time interval, for the Reference and Tumor indexes. The medians of the IMAX values for Region 1 and Region 2 at 4 weeks presented statistically significant differences (p=0.03).

Table IV. The evolution of the medians of the TIC parameters as they were evaluated for group B of experimental animals in various reference and control areas, at 4 and 6 weeks. Values are shown as median (25th percentile – 75th percentile).

<table>
<thead>
<tr>
<th></th>
<th>Reference 4 wks</th>
<th>Reference 6 wks</th>
<th>Tumor 4 wks</th>
<th>Tumor 6 wks</th>
<th>Region 1 4 wks</th>
<th>Region 1 6 wks</th>
<th>Region 2 4 wks</th>
<th>Region 2 6 wks</th>
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<td><strong>TTP (s)</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minimum</td>
<td>7.47</td>
<td>5.34</td>
<td>12.96</td>
<td>14.38</td>
<td>12.01</td>
<td>14.06</td>
<td>16.16</td>
<td>12.1-27.68</td>
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<tr>
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<td>12.09</td>
<td>31.3</td>
<td>24.14</td>
<td>19.55</td>
<td>15.53</td>
<td>10.61</td>
<td>10.9-16.76</td>
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<td>Maximum</td>
<td>29.06</td>
<td>23.87</td>
<td>42.92</td>
<td>33.35</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Minimum</td>
<td>5.45</td>
<td>5.13</td>
<td>7.82</td>
<td>10.17</td>
<td>8.86</td>
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</tr>
<tr>
<td>Minimum</td>
<td>12.07</td>
<td>26.29</td>
<td>16.8</td>
<td>31.41</td>
<td>28.93</td>
<td>15.54</td>
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<td>34.39</td>
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<td>43.51</td>
<td>40.47</td>
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<tr>
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<td>59.49</td>
<td>44.13</td>
<td>161.5</td>
<td>51.1</td>
<td>912.2</td>
<td>233.2</td>
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<tr>
<td><strong>IMAX (%)</strong></td>
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<td></td>
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<tr>
<td>Minimum</td>
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<td>0.3</td>
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<td>100</td>
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<td>100</td>
<td>842.8</td>
<td>202.9</td>
<td>912.2</td>
<td>233.2</td>
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Discussion

The imaging techniques currently used to establish tumor progression and the therapeutic response, using animal experimental models, are represented by: magnetic resonance imaging (MRI), positron emission computed tomography (PET-CT), single-photon emission computed tomography (SPECT), computed tomography (CT), CEUS and optical techniques. In oncology, the application of these methods varies from volumetric quantification to the quantification of functional parameters like tumor vascularity and permeability, cellular proliferation and tissue hypoxia [24].

The present study was focused in the first phase on achieving an ultrasonographic examination protocol for experimental rats, using a high frequency ultrasonographic system. The information acquired during this phase represented the basis for the validation of ultrasonographic techniques applicability on an experimental tumor model, focusing initially on CEUS potential benefits in the characterization of tumor vasculature.

The catheterization of the tail vein was described as a reference method by several authors [25,26]. Nevertheless and in spite of a careful application of the method, our research team did not succeed to establish a viable and reproducible administration line. The difficulty of the approach was caused by the rigidity of the skin and the reduced vascular caliber. The few cases in which the placement of the catheter was successful proved to be also failures due to the displacement of the catheter or the rupture of the vessel consecutive to fine movements of the examiner’s hand or of the tail of the subject.

The intracardiac administration of second generation contrast agents after transthoracic US guided catheterization was not described in the previous published studies [25-28]. Some authors considered the heart catheterization as a terminal method [29]. In the present study, the cardiac approach offered a safe and reproducible method of catheterization. The factor that contributed to the success of this approach was the use of the ultrasonographic guidance. The losses of the subjects using this method for CA administration were determined by the displacement of the tip of the catheter in circumstances of accelerated heart rate. The discrepancies observed between the two methods of CA administration are in favor of the cardiac catheterization.

The characteristics of the tumors, as they were observed in B mode US changed over time as the masses increased in size and thus became more inhomogeneous (see table 2). The echogenicity of the lesions remained rather stable, being a little more hyperechoic than the subjacent muscles. The tumor volume changed significantly during the 2 weeks interval, the lesions did not present spontaneous regression or stagnation and this represented the logical proof of the viability of the tumor cells implantations.

During the CFM exploration, in the advanced phases, a change of the vascularity was noticed. In the initial phase the vessels were predominantly in the periphery, while in the late phase the vascularity was mixed or central.

CEUS is an exploration method that was introduced in practice rather recently. It is a real-time exploration, it is rather inexpensive and it is easily reproducible since it does not use radiation exposure [30]. The new generation of US equipments, like the one used in this study (Vevo 2100), presents the benefit of using small size linear transducers, adapted for the use on experimental animals. The equipment is capable of operating in a suitable mode for the contrast exam and has the capacity to obtain unarchived data which are adequate for the quantitative analysis of the signal. Using the non-linear features of the microbubbles leads to an increase of their detection even when the CA concentration is low [31]. All of these represent the premises for the acquisition of quantifiable, qualitative and quantitative, functional vascular data.

The character of the CA uptake, as observed by CEUS in our study group, was variable. During the initial phase (4 weeks) a peripheral uptake with a slow centripetal progression was predominant. In the late phase (6 weeks) a mixed uptake was predominant, where the peripheral uptake was associated with the central one. The centripetal progression of the uptake was faster than in the initial phase. These features are most likely determined by vascular recruitment as well as by the development of the circulatory bed of the tumor during the interval between examinations. These observations are sustained by the experience of other research teams and show the validity of our experimental model [2].

The quantitative analysis of the CA progression into the tumor is realized using TIC. These are graphic representations of the CA dynamics in a ROI in a given time. The length of an exploration may be unlimited, but an evaluation for as long as the CA is present in the blood stream is sufficient. The mode of penetration, the velocity of uptake in the circulatory bed and the length of CA stagnation represent detectable and quantifiable elements obtained by this procedure and are directly correlated with the inflamed or normal character of the neoplastic circulatory bed [14]. In the present study, the analysis of the TIC parameters showed significant differences between the TTP of the tumors compared with the reference area. Nevertheless there were no statistically significant differences between the TTP of the tumors at 4 weeks.
and at 6 weeks, even though the median of these values changed in the disadvantage of the second moment of examination. The muscle tissue chosen as reference did not present statistically significant differences between the two moments of exploration and it was not influenced by the development of the tumor. This feature sustains the use of this tissue as reference during this study.

The advantages of US compared with the other radio-imaging techniques are in conjunction with the versatility, capability, safety and economical feasibility of this method [32]. The use of high frequency systems (21 MHz) for the examination of the Walker 256 tumor presents the advantage of an excellent visualization of the tumor tissue and of the surrounding structures [33].

But US has limitations as well. One of the most important is related to the fact that this method requires a well-trained examiner in order to acquire high quality, reproducible images. Even though the images obtained through this exploration may be used to extract information about structure and function, US is currently inferior to other techniques when it comes to metabolic and biochemical data [32].

Our observations on this group of experimental animals have a preliminary character. We believe that the work protocol can be improved by using contrast agents optimized for preclinical studies and high frequency examinations (ex: VeVo MicroMarker, Bracco research SA). The median diameter of these microbubbles is 2.3-2.9 μm, which allows a more detailed analysis of the microcirculation. We found that the increased coagulability and accelerated heart rate of the Wistar albino rats represent factors which enhance the difficulty of the procedure. These disadvantages may be partially prevented by using a gas system for anesthesia and by an ECG monitoring of the subjects.

The tumor model presented in this study holds the advantage of being easy to implement in a short period of time, representing a bridge between preclinical studies and clinical research on human subjects. The dynamic exploration is accurate and reproducible. Even though the group of animals in the study is small we consider that the results are encouraging for continuing the research and evaluating microcirculation after it has been exposed to various drugs. Defining a methodology for the contrast enhanced ultrasonographic examination is an important phase of any research that aims the acquisition of data by applying this method on a experimental tumor on rats. Since CEUS evaluation is adequate for the assessment of tumor vascularity on the murine Walker 256 tumor model, the method may become an essential component of that studies which propose the evaluation of tumor vascularity in vivo on an animal model in connexion with the development of new antitumoral therapies [34,35].

The novelty of this study consists in the successful intracardiac administration of the CA, the use of a high frequency system and of US contrast agents developed for human usage and the quantification of the TIC parameters.

Conclusions. Walker 256 tumor has the advantage of a quick achievement of an experimental model, while its implantation on the thigh facilitates the ultrasonographic examination. Intracardiac administration of the CA is a rather easy applicable procedure in the context of the sono graphic guidance. Contrast enhanced ultrasonography using microbubbles represents a viable method in establishing the degree of tumor vascularization and identifying hypovascular/necrotic areas on the experimental rat. The present study sustains our team’s efforts in the development of protocols for future studies that will identify adequate CEUS parameters for the characterization of the antiangiogenic therapeutic response.

Conflicts of interests: none.

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