Study on breast cancer animal model of tumor-micro vessel variation before and after the chemotherapy by contrast enhanced ultrasound quantitative analysis

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Abstract: Aim to discuss whether the contrast enhanced ultrasound (CEUS) can effectively monitor the efficacy on neoadjuvant chemotherapy of breast cancer or not by analyzing the indicators on chemotherapy CEUS and breast cancer tumor biology, especially tumor microcirculation indicator on animal mode. Human breast cancer cell lines MCF-7 are planted under the skins of nude mice. By simulating clinical neoadjuvant chemotherapy regimen periodically inject CMF (cyclophosphamide, methotrexate and fluorouracil) into the experimental group, and normal saline into the control group. Then detect the data from CEUS and record the parameters: maximum intensity (IMAX), rise time (RT), time to peak (TTP) and mean transit time (mTT). Execute animal after CEUS, obtain tumor biological indicator and record parameters: micro vessel density (MVD), vascular endothelial growth factor receptors 1/2/3/4 (VEGFR-1/2/3/4) and tumor cells. In the aspect of tumor biological indicator, the experimental group after the first drug delivery: inter- and intra-group comparisons of VEGFR-1/4drop significantly. The experimental group after the second drug delivery: inter- and intra-group comparisons of MVD, VEGFR-1/3/4drop significantly. In the aspect of parameters on tumor CEUS, the experimental group after the first drug delivery: inter- and intra-group comparisons of IMAX drop significantly. The experimental group after the second drug delivery: inter- and intra-group comparisons of IMAX decrease steeply; while inter-and intra-group comparisons of TTP rise significantly. There are great changes about the intra-group comparisons of the number of tumor cells before and after the experiment. In the process of chemotherapy, it maintains the consistency of the changes of CEUS parameters IMAX and TTP, tumor microcirculation indicators MVD and VEGFR-1/3/4 and tumor cells. So CEUS has a potential to make an early prediction on the efficacy of neoadjuvant chemotherapy.

Keywords: Contrast enhanced ultrasound, breast cancer, chemotherapy, animal model.

INTRODUCTION

Neoadjuvant chemotherapy (NC) of breast cancer has been broadly used, but efficacy prediction is lack of efficient imaging modalities. Although traditional two-dimensional ultrasound can be used to detect the size changes of tumor before and after the chemotherapy, it is hard to predict the final efficacy (Keune *et al.*, 2010; Marinovich *et al.*, 2012). Tumor facilitates a large amount of neovascularization, which is the basis of growth, invasion and transfer of tumor (Weidner, 2004; Foote *et al.*, 2005). Because of the pure-blooded pool perfusion characteristics of ultrasound contrast agent, we attempted to observe the changes of blood supply in tumor before and after the chemotherapy by contrast enhanced ultrasound (CEUS) so as to achieve the early prediction of tumor efficacy.

MATERIALS AND METHODS

Preparation on breast cancer nude mice transplanted model

SPF Balb/c nude mice, SPF level, female, 4 weeks old, SLRC Laboratory Animal Co. Ltd. Animals are reared in barrier system in which temperature is 22-24□and relative humidity is 45%-65%; interior lighting belongs to artificial simulation that alternates light and dark in 12 h, and its illumination is 200-300lx; air change rate in the room is 20 times for an hour, and ammonia concentration is 14mg/m³ below. All tested animals are fed with 60Co irradiated pellet feed and sterile water. Human breast cancer cell line MCF-7 is from central lab of Fudan University Cancer Hospital. Cells under log chase of growth are chosen to make cell suspension whose concentration is about $2*10^7$/m. 0.3 ml cell suspension is injected and inoculated into the right breast pads of every nude mouse, and nude mice are induced to produce breast cancer. Continuously observe for 1 week after inoculation. And it will be considered as tumor if some indicators appear like tumor nodule in subcutaneous inoculation position and hard texture. After 4 weeks, we should make a further experiment when the diameter of tumor body reaches around 2.0cm.

Experiment grouping

Tumor-bearing nude mice are randomly divided into experimental group and control group with 30 mice in each group. Each group can be divided into 3 subgroups with 10 mice in each subgroup in the light of time nodes of drug delivery: group that mice were executed before

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Table 1: Physical indicators of tumor

	Before Chemotherapy			Chemotherapy Once			Chemotherapy twice		
	Experimental group	Control group	P	Experimental group	Control group	P	Experimental group	Control group	P
Weight (g)	20.7 ±0.35	20.65 ±0.47	.837	19.15 ±0.24	21.85 ±0.34	.000	15.55 ±0.72	23.4 ±0.84	.000
Tumor Volume (mm^3)	37.25 ±13.18	38.32 ±11.67	.962	51.43 ±17.18	140.13 ±41.03	.000	34.36 ±26.99	382.77 ±112.99	.000

Table 2: Microcirculation indicators of tumor

	Before Chemotherapy			Chemotherapy Once			Chemotherapy twice		
	Experimental group	Control group	P	Experimental group	Control group	P	Experimental group	Control group	P
MVD	17.80 ±2.77	17.81 ±2.21	.893	16.46 ±1.37	18.03 ±2.76	.138	13.30 ±1.51	17.96 ±2.34	.000
VEGFR-1	264.01 ±5.22	263.77 ±5.18	.956	205.66 ±38.82	264.29 ±52.87	.008	34.01 ±6.34	268.47 ±54.94	.000
VEGFR-2	211.46 ±94.78	207.6 ±92.15	.837	188.3 ±84.4	209.76 ±43.33	.558	165.17 ±44.6	212.2 ±98.66	.202
VEGFR-3	159.54 ±59.65	155.11 ±59.99	.911	116.28 ±41.37	158.64 ±56.08	.069	89.06 ±12.58	159.58 ±59.64	.003
VEGFR-4	141.27 ±31.75	140.99 ±31.77	.906	114.75 ±25.63	141.07 ±21.35	.033	45.54 ±14.34	143.43 ±31.83	.000

Table 3: CEUS indicators

	Before Chemotherapy			Chemotherapy Once			Chemotherapy twice		
	Experimental group	Control group	P	Experimental group	Control group	P	Experimental group	Control group	P
IMAX	366.19 ±68.57	366.24 ±69.48	.999	256.92 ±53.10	366.54 ±71.29	.001	76.96 ±23.49	364.55 ±92.49	.000
RT	13.4 ±2.17	13.5 ±2.55	.838	14.9± 3.14	13.5 ±2.22	.215	17± 3.16	13.7 ±2.4	.005
TTP	15.85 ±3.22	16.1 ±3.2	.896	17.48 ±3.29	16.02 ±2.75	.427	22.52 ±6.95	16.12 ±3.56	.001
mTT	43.69 ±6.26	43.65 ±6.3	.998	43.93 ±8.69	43.6 ±7.62	.925	44.78 ±5.94	42.63 ±10.85	.540

chemotherapy on the first day, group that mice were given chemotherapy once and executed on the 7th day, group that mice were given chemotherapy twice and executed on the 14th day. Before execution, mice in each subgroup should be weighed, and then the longest and shortest diameters were measured. Reduction formula of tumor volume the longest diameter*the shortest diameter $^2/2$.

Chemotherapy regimen

Drug regimen of experimental group CMF: cyclophosphamide (C), methotrexate (M), fluorouracil (F). And drug regimen is strictly controlled according to Lloyd experimental design (Lloyd, 1974). Each dosage is 1/3 $LD_{10.}$ The dosages of each drug LD_{10} are provided specifically as follows (Lindén, 1989; Inaba *et al.*, 1989; Seigers, *et al.*, 2015): cyclophosphamide 100mg/kg, methotrexate 27mg/kg and fluorouracil 140mg/kg. The experimental group is injected drug into abdomen at 1th and 7th day. The control group is injected normal saline into abdomen at corresponding time nodes.

CEUS technique

All experimental animals were examined by CEUS before execution. Use MyLab90 color Doppler ultrasonic diagnostic apparatus from Esaote Company with high frequency probe LA523 from 4-13MHz and Sonovew ultrasound contrast agent from Bracco Company. Each nude mouse is injected 0.005ml to caudal vein. Immediately observe the radiography and record data after injection. After finishing radiography, implant located needle under probe to determine the observing region. Data of CEUS is made an off-line analysis by using analysis software Sonoliver from Tomtec Company. What parameters of CEUS are gained are: maximum

intensity of tumor perfusion (IMAX); required time on 10%-90%maximum intensity, namely rise time (RT); time to peak (TTP); time that maximum intensity of nidus decreases by half, namely mean transit time (mTT).

Histological analysis

All nude mice are executed by cutting off necks after the implantation of located needle. After execution, dig tumor out along the direction of probe and take located needle about 5mm in thickness. The length includes tissues of all tumors of CEUS. Then embed in paraffin and cut into slices after fixation. Later detect micro vessel density (MVD) and expression of CD-31 by adopting immunohistochemistry. Rabbit-anti-rat CD-31 monoclonal antibody is used in the first antibody, while goat-anti-rabbit IgG is adopted in the second antibody; vascular endothelial growth factor receptors 1/2/3/4 (VEGFR-1/2/3/4) apply immunohistochemistry to detect the expression of VEGFR-1/2/3/4. Rabbit-anti-rat VEGFR-l/2/3/4 polyclonal antibody is used in the first antibody, while second antibody adopts goat-anti-rabbit IgG. Final sections are done to the tumor specimen in the subgroups before chemotherapy and after the second chemotherapy and tumor cells with high power lens are counted under a light microscope.

STATISTICAL ANALYSIS

Use SPSS17.0 software. And data is expressed by $\bar{x} \pm S$. Data can be compared between subgroups from experimental group and control group in the same time node, while three subgroups respectively from experimental group and control group can be compared as intra-group comparison. Statistical processing method is that all single factors are made analysis of variance with factorial design, and if there occurs statistical difference (grouping or time), it should be further compared in pairs. $p<0.05$ has statistical significance.

RESULTS

Physical indicators of tumor

Physical indicators of tumor include weight of tumor-bearing nude mice and volume size of tumor as shown in table 1. After the first drug delivery: there are significant differences in the comparisons of weight inter-group ($p=0.000<0.01$) and intra-group ($p=0.000<0.01$); inter-group comparison on tumor volume ($p=0.000<0.01$) has remarkable difference, while intra-group ($p=0.539 > 0.05$) has no obvious variance. After the second drug delivery: there are significant differences in the comparisons of weight inter-group ($p=0.000<0.01$) and intra-group ($p=0.000<0.01$); inter-group comparison on tumor volume ($p=0.000<0.01$) has dramatic difference, while intra-group ($p=0.46>0.05$) has no obvious variance.

Microcirculation indicators of tumor

Microcirculation indicators of tumor include MVD and VEGFR-1/2/3/4 as shown in table 2. After the first drug delivery: there are no difference in the inter-group comparisons of MVD ($p=0.138>0.05$) and VEGFR-3 ($p=0.069>0.05$) and intra-group of MVD ($p=0.205>0.05$) and intra-group of VEGFR-3 ($p=0.064>0.05$); inter-group comparisons on VEGFR-1 ($p=0.008<0.01$) and VEGFR-4 ($p=0.033<0.05$) drop steeply, so do intra-group comparisons of VEGFR-1 ($p=0.007<0.01$) and VEGFR-4 ($p=0.021<0.05$). After the second drug delivery: inter-group comparisons of MVD ($p=0.000<0.01$) and VEGFR-3 ($p=0.003<0.01$) decrease significantly, so does intra-group comparison of MVD ($p=0.004<0.01$). Besides, comparing with intra-group contrast before chemotherapy, VEGFR-3 drops remarkably, while it has no difference comparing with intra-group contrast with chemotherapy once ($p=0.239>0.05$); inter-group comparisons of VEGFR-1 ($p=0.000<0.01$) and VEGFR-4 ($p=0.000<0.01$) drop significantly, which are shown in figs. 1-4. fig. 5-8 shows the changes of microcirculation indicators of tumor under microscope.

CEUS indicators

CEUS indicators include IMAX, RT, TTP and mTT, which can be seen in table 3. After the first drug delivery: inter- ($p=0.001<0.01$) and intra-group ($p=0.001<0.01$) comparisons of IMAX drop significantly; but inter- ($p=0.427>0.05$) and intra-group ($p=0.376 > 0.05$) comparisons of TTP have no remarkable difference. After the second drug delivery: inter- ($p=0.000<0.01$) and intra-group ($p=0.001<0.01$) comparisons of IMAX drop steeply; while inter- ($p=0.427>0.05$) and intra-group ($p=0.376>0.05$) comparisons of TTP increase sharply, which can be seen in fig. 9 and 10.

Pathological result

Count under high power lens. Densities of tumor cells in experimental group are 464.92±60.8500 per high power field before chemotherapy and 136.99±17.30 per high power field after second chemotherapy. So tumor cells decrease significantly after chemotherapy ($p=0.000 <0.01$).

DISCUSSION

Neoadjuvant chemotherapy is an important therapeutic means for breast cancer. Because reactions of nidus to different medicines are not consistent, it is a big problem how to predict curative effect and guide the clinical treatment by imaging method at early chemotherapy time (Keune *et al.*, 2010; Marinovich *et al.*, 2012). The existing researches (Weidner, 2004; Foote *et al.*, 2005) show that the growth, development and degeneration of tumor are related to its growth of blood vessel, so we consider predicting tumor outcome by observing the changes of tumor blood supply. The contrast-enhanced

ultrasound technique (Xu, *et al.*, 2015) forms tumor imaging by micro-bubble imaging in blood pool circulation system, and a large number of parameters can be analyzed quantitatively. According to the reports by Palmowski *et al.* (2008) and Cao *et al.* (2012), the curves of ultrasound contrast have significant changes before and after neoadjuvant chemotherapy. However, the result is lack of adequate quantization parameters. And it has not been studied about the relations between early changes and curative effect of tumor chemotherapy.

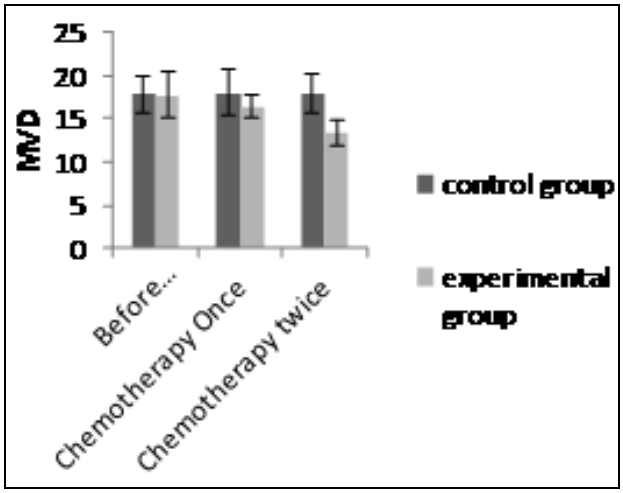

Fig. 1: The changes of MVD

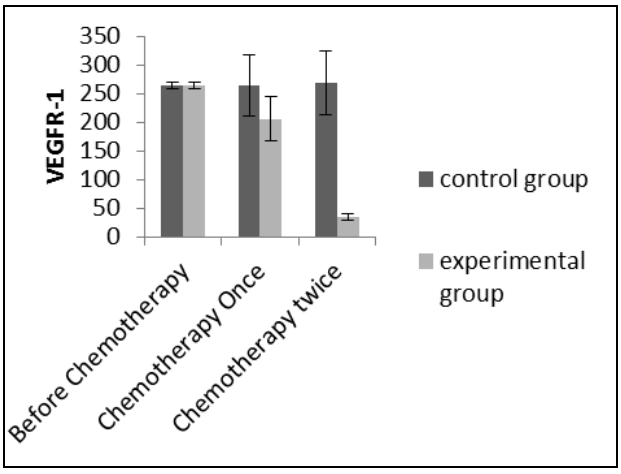

Fig. 2: The changes of VEGFR-1

recent reports by Wang *et al.* (2013) and Chen *et al.* (2015), after the usage of chemotherapy drugs alone such as adriamycin or cisplatin, the microcirculation indicators of tumor have changed and parameters of contrast-enhanced ultrasound also have significant changes. This paper aims to study simulated clinical chemotherapy scheme, the changes situation on indicators of tumor microcirculation and contrast-enhanced ultrasound after medication, as well as analyze the relationship between them.

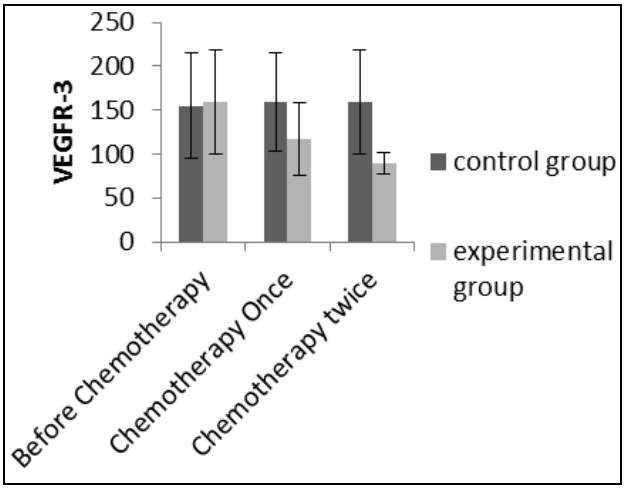

Fig. 3: The changes of VEGFR-3.

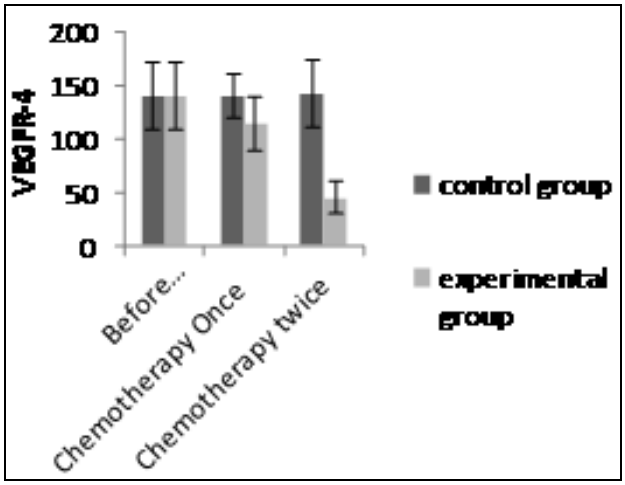

Fig. 4: The changes of VEGFR-4

According to the reports (Pysz *et al.*, 2011; Forsberg *et al.*, 2011; Rix *et al.*, 2012; Pysz *et al.*, 2012) with decreases of microcirculation indicators of tumor by the usage of antiangiogenic agents in animal tumor model, parameters of CEUS appear changes with different degrees and are related to curative effect of antiangiogenesis in some research results (Zhu *et al.*, 2011; Seshadri *et al.*, 2011; Wang *et al.*, 2015). However, most of chemotherapy drugs are used in clinic to suppressing tumor growth by inhibiting the synthesis of DNA and RNA of tumor cell and the mechanism is different from the antiangiogenesis drugs. In the light of

The research results indicate that tumor MVD in the intra and inter- groups drop significantly after the second chemotherapy, which coincides with past reports; we have a further discovery that VEGFR-1/4 in the intra and inter-groups fall steeply after the first chemotherapy, which prompts that MVD is more sensitive by comparison between them for indicators of microcirculation; by contrast, VEGFR-3 in the inter-group at the second chemotherapy appears significant decrease, but it in the intra-group drops dramatically by comparing with it before chemotherapy. However, there are no significant changes of VEGFR-3 in inter- and intra- groups after the

first chemotherapy, which suggests that the sensibilities of these indicators are lower than those of MVD. The number of tumor cells is down to 70% above. It belongs to grade 3 partial responses according to pathology MP (Ogston *et al.*, 2003). The decrease of infiltrating tumor cells between 30%-90% belongs to moderate decrease. It is found that not only does tumor cell inactivate after chemotherapy, but tumor has a sharp change on microcirculation.

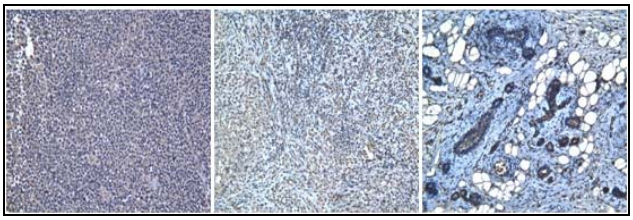

Fig. 5: The changes of MVD under microscope. The graphs from left to right are sections of three subgroups in experimental group before chemotherapy, chemotherapy once and chemotherapy twice.

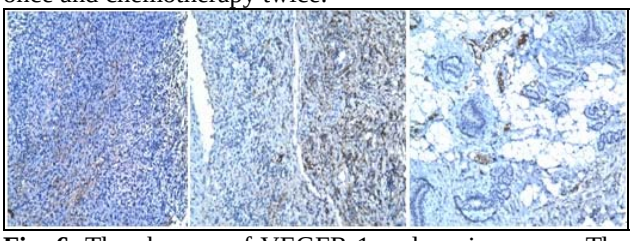

Fig. 6: The changes of VEGFR-1 under microscope. The graphs from left to right are sections of three subgroups in experimental group before chemotherapy, chemotherapy once and chemotherapy twice.

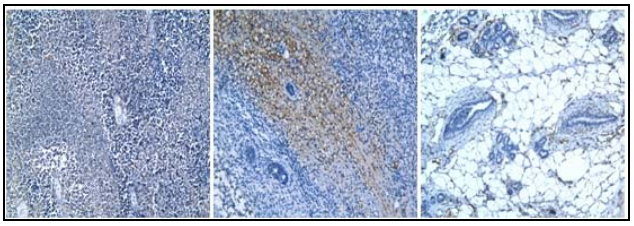

Fig. 7: The changes of VEGFR-3 under microscope. The graphs from left to right are sections of three subgroups in experimental group before chemotherapy, chemotherapy once and chemotherapy twice.

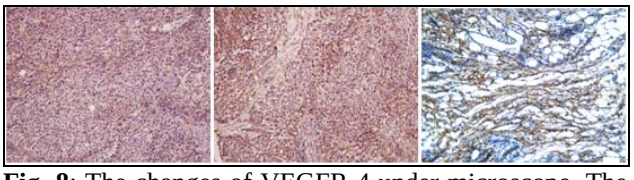

Fig. 8: The changes of VEGFR-4 under microscope. The graphs from left to right are sections of three subgroups in experimental group before chemotherapy, chemotherapy once and chemotherapy twice.

The research results indicate that tumor VEGFR-2 in the intra and inter- groups have no significantly changes after the first and the second chemotherapies. By the report of Guo *et al.* (2010), VEGFR-2 is generally recognized to have a principal role in mediating VEGF-induced responses in breast cancer, and can be regulated by inhibitors to treat tumor. However, in our research, there are conventional thermo therapies drugs used, which can't inhibit VEGFR-2 directly. Also, report by Meunier-Carpentier *et al.* (2005) shows that expression of VEGFR-2 is not correlated with tumor prognosis, which is coincidence with our results.

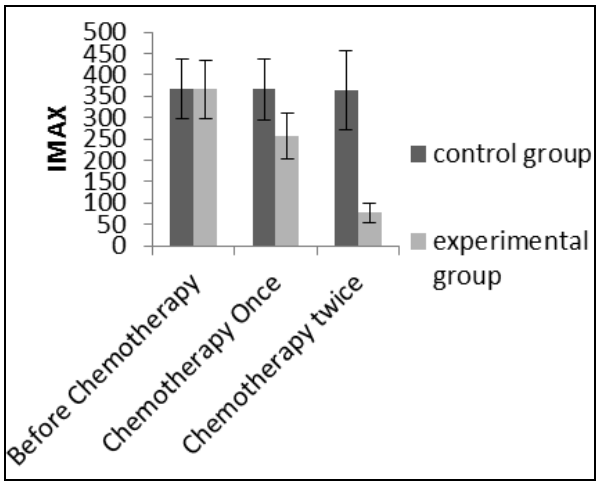

Fig. 9: The changes of IMAX

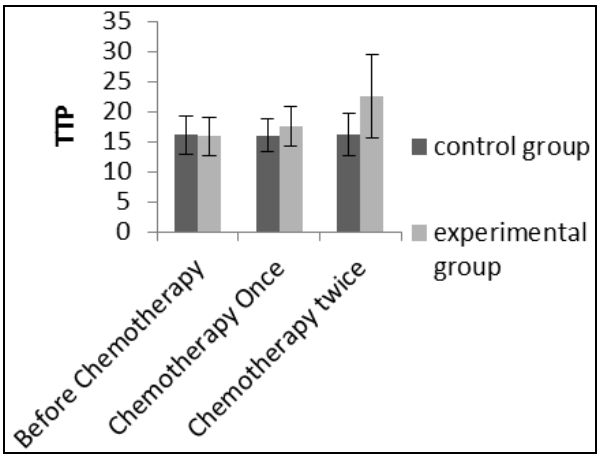

Fig. 10: The changes of TTP

Past studies on parameters of CEUS aimed to area under curves (AUC). It can be learnt in our analysis that the changes of AUC depends on IMAX, and the size IMAX affects the height of curve to change AUC. The study result shows that IMAX has great change after the first chemotherapy, which is consistent with the decrease of AUC in the existing reports. TTP in inter- and intra-groups tend to rise dramatically after the second chemotherapy, which denotes that perfusion time increases after tumor inactivation, which may be the result of destroyed microcirculation.

Tumor cells decrease significantly after the second chemotherapy; there has been a dramatic change about tumor microcirculation indicator, and change of VEGFR-1/4 happens at early stage and is quicker than that of MV

and VEGFR-3; The change of CEUS indicator is still the significant change of IMAX perfusion amount at early stage and quicker than that of TTP. It is supposed that the changes of microcirculation indicator and CEUS indicator have consistency in the process of chemotherapy and are earlier than physical change of tumor, which shows that the partial indicator changes of CEUS have a potential to predict inactivation of tumor cells and the lapse of tumor after the chemotherapy. Figs. 11-13 presents the changes of CEUS curves with the changes of the chemotherapy. The most remarkable change is the increase of IMAX.

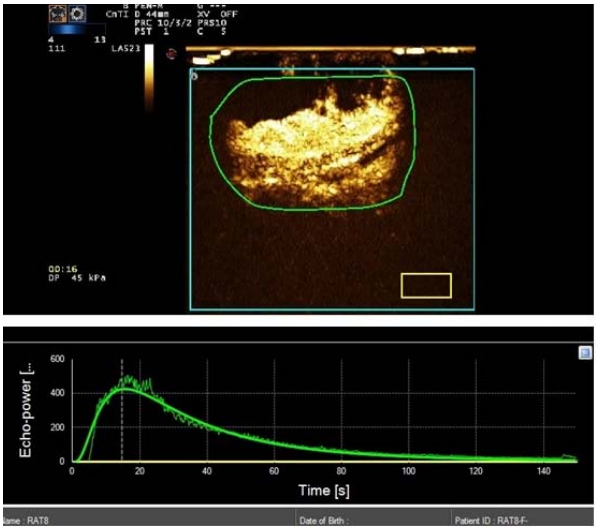

Fig. 11: CEUS of tumor before chemotherapy. The images above and below are an image when perfusion reaches to peak and a perfusion curve respectively. The unsmooth curve is an original perfusion curve. What should be paid attention is that time in figure includes previous period from injection to perfusion rather than TTP. Perfusion area is not totally perfused. And there is necrosis in partial area of tumor.

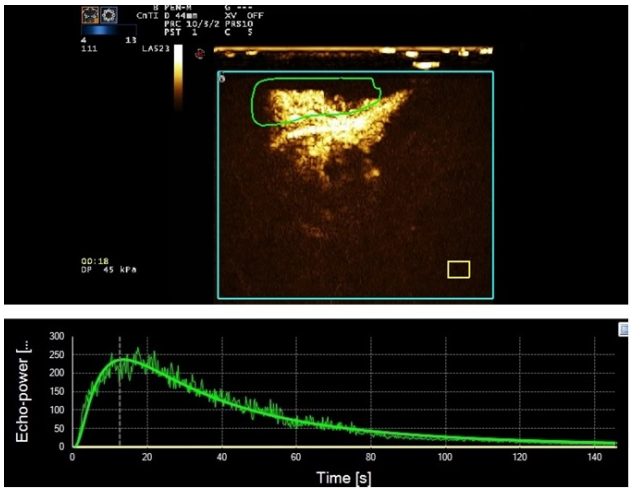

Fig. 12: CEUS of tumor after the first chemotherapy. There is no necrosis in the perfusion area of the image above, and the following curve shows the decrease of perfusion intensity.

The shortcoming in this study is relatively few sample sizes, and only one neoadjuvant chemotherapy scheme has been simulated. MCF-7 cell also cannot embody the breast cancer immunohistochemistry and diversity of genotyping. Therefore, the current results should be proved by making a further clinical research.

CONCLUSION

In the process of chemotherapy, it maintains the consistency of the changes of CEUS parameters IMAX and TTP, tumor microcirculation indicators MVD and VEGFR-1/3/4 and tumor cells. So CEUS has a potential to make an early prediction on the efficacy of neoadjuvant chemotherapy.

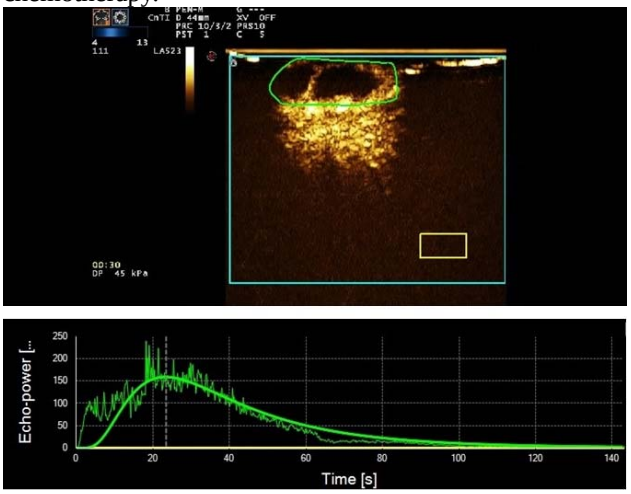

Fig. 13: CEUS of tumor after the second chemotherapy. The above image indicates that tumor perfusion area decreases significantly. There are necrosis in a large area and ring-enhancement around it. The image below shows that perfusion curve intensity drops remarkably. TTP cannot be seen directly, but after injection, the time to peak prolongs obviously.

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