doi.org/10.36721/PJPS.2022.35.5.REG.1467-1471.1

Potential of curcumin loaded nanoparticles in ovarian cancer: Investigation using gynecological color Doppler ultrasound technique

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Abstract: Doppler ultrasonography is a type of medical ultrasonography that uses the Doppler effect to provide images of the movement of tissues and bodily fluids (typically blood) relative to the probe. To determine the potential of curcumin loaded nanoparticles in ovarian cancer which was diagnosed by using the gynecological color doppler ultrasound technique. Curcumin (CRMN) loaded chitosan nanoparticles were formulated using the ionotropic gelation method and characterized for particle size, zeta potential and polydispersity index (PDI). Clinical parameters like serum creatinine, blood serum urea nitrogen, resistance index and peak systolic velocity were evaluated. The drug loading efficiency was found between 11.38 to 17.45% with a particle size of 140-220nm. The zeta potential ranged between 19.12 to 23.14mV. Clinical parameters were found significantly changed when compared with before injection of CRMN-loaded nanoparticles. BUN was increased from 7.11±0.25to 28.27±6.65 mmol/L while SCr was also found to be increased from 52.71±3.14 µmol/L to 312.20±40.41 µmol/L. Collectively, these images of color doppler in animal model demonstrated efficient use in the diagnosis of ovarian cancer. This study confirms the potential of color doppler as an efficient medical imaging tool for ovarian cancer.

Keywords: Ultrasound, color doppler, medical imaging, ovarian cancer, drug loading.

INTRODUCTION

Globally, ovarian cancer is the leading cause of mortality among gynecological malignancies. Debulking surgery and systemic therapy are used to treat ovarian cancer. Ovarian cancer (OC) is the fourth leading cause of cancer death in women in the developed world, accounting for about 150,000 fatalities per year (Chevalier *et al*., 2009). OC is the deadliest of all gynecologic cancers due to its largely asymptomatic nature and advanced illness at the time of diagnosis. Because of a lack of effective screening tests, 75 percent of patients are identified at late FIGO (International Federation of Gynecologists and Obstetricians) stages (III and IV), implying that better approaches for early and specific identification of OC are needed (Platt *et al*., 1993). The current treatment for advanced OC relies on the synergistic effect of combining surgical cytoreduction and chemotherapy; however, new imaging strategies are needed to target microscopic lesions and improve both cytoreductive surgery and patient outcomes, in addition to the fact that chemotherapy resistance is a major challenge in OC management (Webb, 1990). Color doppler ultrasound technique (CDUT) was introduced in 1996 and is widely used in the diagnosis of liver, abdominal and vascular dysfunctions in the US, Europe and Asia. This technique has several advantages over contrast-enhanced magnetic resonance imaging (CEMR) and contrast-enhanced computed tomography (CECT) including no use of radiation and no harmful effects on other organs like thyroid, kidneys, etc., easy application and comfort during use (Kalantarinia, 2009). Conventional grayscale and other techniques are useful for anatomical information and blood flow information in large vessels respectively. These techniques cannot be useful for the evaluation of blood flow in microvessels and tissue perfusion (Bossy *et al*., 2005). Color flow mapping has become a valuable tool in human medicine for investigating the blood flow of placental circulation in normal pregnancy, predicting fetal development retardation and diagnosing different illnesses. Color Doppler ultrasonography has previously been used to measure uterine blood flow and perfusion during late normal pregnancy and in uterine torsion afflicted buffaloes but the blood flow to the main uterine artery following detorsion of the uterus has not been assessed (Nilsson, 2004). Doppler ultrasonography is a type of medical ultrasonography that uses the Doppler effect to provide images of the movement of tissues and bodily fluids (typically blood) relative to the probe. The speed and direction of a given sample volume, such as blood flow in an artery or a jet of blood flow through a heart valve, may be calculated and seen by computing the frequency shift of that sample volume. Color Doppler, often known as color flow, is a device that measures the amount of color Doppler and is a color-coded representation of velocity. To display duplex ultrasonography pictures, color Doppler images are usually merged with grayscale (B-mode) images, allowing for simultaneous viewing of the anatomy of the region (Johnson *et al*., 2005). Flow ultrasound images, whether color flow or spectral Doppler, are derived primarily from movement data. A sequence of pulses is sent in ultrasonic

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scanners to detect blood movement. From one pulse to the next, the echoes from stationary tissue are the same. Moving scatterer echoes have minor variations in the time it takes for the signal to be returned to the receiver. These discrepancies can be quantified in terms of a direct time difference or, more often, a phase shift from which the Doppler frequency can be calculated. The images are then converted into a color flow display or a Doppler sonogram. The CDUT technique has been widely used for the diagnosis of focal liver lesions, and kidney dysfunctions including renal nephropathy, cystic lesions, kidney tumors and cortical necrosis (Chang *et al*., 2016; Yang *et al*., 2019). So, considering all these potentials of CEUS in the diagnosis of various diseased conditions we have studied the potential of curcumin loaded nanoparticles in the treatment of ovarian cancer using CDUT as a potential medical imaging tool in a rabbit model.

MATERIALS AND METHODS

Materials

Curcumin (CRMN) was obtained as a gift sample from Baoji Guokang Bio-Technology Co., Ltd. (Baoji, Shaanxi, China). Tripolyphosphate (TPP), Chitosan (CS), and Poloxamer (PLR) were obtained as a gift sample from Sigma Aldrich, USA. Acetic acid was donated by Shanghai Chemical Co. (Shanghai, China).

Preparation of CRMN-loaded nanoparticles

CS-CRMN nanoparticles were formulated using the ionotropic gelation method using TPP as a crosslinking agent. CS (25- 100mg) was dissolved in 3% v/v acetic acid solution (20ml) under continuous magnetic stirring for nearly 45 minutes. After complete solubilization of CS, Tween 60 was added at the concentration of 0.1 to 0.4 ml under continuous magnetic stirring for 45 minutes. CRMN (3.5 mg/ml) was dissolved separately in ethanol and added dropwise to the polymeric solution of CS using a syringe fitted with a needle. TPP (0.2 to 0.8mg) previously dissolved in 5 ml of purified water was added dropwise to result in dispersion. This dispersion was kept overnight for crosslinking of CS and TPP. The formula composition is presented in table 1. The resulted in nanoparticulate suspension was centrifuged (at -50-70^{o}C; 30,000 RPM) to separate the nanoparticles from the solution. The separated nanoparticles were freeze-dried using trehalose as a cryoprotectant (Yadav *et al*., 2018). The resulted nanoparticles were used for further characterization.

Animals

Female healthy rabbits (n=24) weighing 2.5 to 3.5kg were used in this research work. The animals were kept under normal environmental conditions and a healthy diet was provided two times a day for 15 days. Ovarian cancer was induced in all animals as per the procedure reported in the literature. These animals were divided into two groups each containing 12 animals. All animal handling procedures and experiments were done with the prior permission of the institutional animal ethical committee. Group I was administered with CRMN-loaded nanoparticles and the other group was considered a control group. The anesthesia was induced by IM injection of Midazolam (0.05 mg/kg) + Buprenorphine (0.03mg/kg) + Ketamine (10 mg/kg) and further analysis was done.

Color Doppler examination

The most significant method for the morphological examination of OC with the use of Doppler and color doppler to examine mass vascularization is pelvic transvaginal sonography (TVS) in combination with abdominal and pelvic transabdominal sonography. However, because of the large number of false-positive results, the technique's accuracy is restricted. Color Doppler imaging and pulsed Doppler spectrum were used to assess ovarian tumor blood flow, analyses blood vessel distribution, and quantify blood flow velocity waveforms. These factors improve the sensitivity and specificity of ovarian tumor ultrasonography assessment. For comparison examinations were also done with conventional ultrasound technique and observations were recorded (Dong *et al*., 2012).

Characterization of CRMN-loaded nanoparticles

Particle size, Zeta potential and polydispersity index (PDI)

The developed NPs were characterized for particle size and zeta potential using a Malvern particle size analyzer. The nanoparticulate suspension was diluted 10 times using purified water and sonicated for 20 minutes to get a properly dispersed nanoparticulate suspension. This suspension was loaded in a particle size analyzer at room temperature and particle size, zeta potential, and PDI were determined (Hu and Luo, 2021).

CRMN loading efficiency

The CRMN concentration in supernatant solution was determined after centrifugation and samples were analyzed by the HPLC method. The loading efficiency was determined by using the formula presented below (Kurakula *et al*., 2021).

% DLE = Target Loading - Unloaded NLX / Target Loading X 100

Evaluation of clinical parameters

The blood samples were withdrawn from animals before and after each color doppler examination and color doppler in the diagnosis of OC was compared with various clinical parameters like serum creatinine, blood serum urea nitrogen, resistance index, and peak systolic velocity.

Table 1: Formula composition of CRMN-loaded CS nanoparticles

Formulations	F1	F2	F3	F4
CRMN (mg)	50	50	50	50
Ethanol (ml)	1	1	1	1
Chitosan (mg)	25	50	75	100
3% Acetic acid (ml)	20	20	20	20
Tween 60 (ml)	0.1	0.2	0.3	0.4
TPP (mg)	0.2	0.4	0.6	0.8
Purified water	q.s.	q.s.	q.s.	q.s.

Table 2: Characterization of CRMN-CS-NP

Code	DL (%)	PSD (nm)	Zeta potential(mV)	PDI
F1	11.38	140	19.12	0.21
F2	13.24	160	19.89	0.27
F3	15.26	195	20.27	0.19
F4	17.45	220	23.14	0.20

Table 3: Comparative clinical parameters before injection, after 6hr and 24 hours of injection.

Index	Before injection	8 hours after injection	24 hours after injection
BUN (mmol/L)	7.11±0.25	14.70±1.15	28.27±6.65*
SCr (µmol/L)	52.71±3.14	89.19±2.12	312.20±40.41*
Resistance index (RI)	0.51±0.21	0.55±0.11	0.90±0.18*
Peak systolic velocity (PSV) (cm/s)	69.28±5.14	72.14±8.45	89.21±7.18*

Histopathological analysis

After color doppler examination 10 animals were sacrificed and histopathological analysis was performed. The ovaries were removed and kept in 10% formalin solution for 24 hours. The kidneys were sectioned in 3 mm slice thickness. Histological analysis was done after staining with eosin and hematoxylin.

STATISTICAL ANALYSIS

The statistical analysis of the results obtained in this study were analysed by using a statistical kit of SAS (version 9.0; SAS Institute, Inc., Cary, NC). Analysis of Variance (ANOVA) test was performed and the significance of degree was taken as p-value <0.005.

RESULTS

The drug loading efficiency was found between 11.38 to 17.45 % with particle sizes ranging from 140-220 nm. The PDI values showed that the formulated nanoparticles had uniform size distribution within nanoparticulate suspension (See table 2). Also, the zeta potential values (19.12 to 23.14 mV) of the suspension confirmed the physical stability of the nanoparticles. As can be seen from table 3 clinical parameters were found significantly changed when compared with before the injection of CRMN-loaded nanoparticles. BUN was increased from 7.11±0.25to 28.27±6.65 mmol/L while SCr was also found to be increased from 52.71±3.14 µmol/L to 312.20±40.41 µmol/L. Similarly, RI was also increased from 0.51±0.21 to 0.90±0.18 and PSV from 69.28±5.14 to 89.21±7.18 cm/s. The results were found to be statistically significant ($p< 0.05$). The images before and after injection of CRMN-loaded nanoparticles were captured (see figs. 1 and 2). Before induction of OC, all biological parts of the ovary were found to be normal and these are more distinct observed in images captured by color doppler technique as compared to conventional ultrasound technique (fig. 1). In color doppler, we also observed continuous and slow enhancement in ovarian arteries and other cellular and tissue vasculature (See fig. 2). Further histopathological study was conducted on 10 animals by staining with eosin and hematoxylin. The analysis was performed before injection, after 8 hrs., and 24 hrs. after injection. As can be seen from fig. 3 significant changes have been observed after the injection of glycerin. fig. 3A shows the abnormal biological appearance of the ovary with a loss of organized structure. But after injection at 6 hours of time point slightly organized structure was observed. The scenario became very excellent at 24 hours of injection with a complete gain of cellular structure.

DISCUSSION

Physico-chemical properties of nanoparticles

The nanoparticles were prepared by using chitosan polymer due to their excellent biocompatible potential in drug delivery systems (*Olaru et al.,* 2018). The nanoparticles were formed due to the interaction between CS (Positive charge) and TPP (Negative charge) (Liu *et*

al., 2012; Pramanik *et al.,* 2021). The ionotropic gelation method was found to be a suitable method for the preparation of CRMN-CS-NP (Khairnar *et al.,* 2019). The drug loading efficiency was found between 11.38 to 17.45 % with particle sizes ranging from 140-220 nm. The loading efficiency was directly associated with the concentration of CS used in formulation (Mokale *et al.,* 2014). Increasing the CS concentration helped in maximum loading of the CRMN. Also, the zeta potential values (19.12 to 23.14 mV) of the suspension confirmed the physical stability of the nanoparticles. The surface characteristics of the nanoparticles were studied using FESEM and it was found that the nanoparticles (F4) were found to be spherical, smooth, and without a crack in nature. From all these observations F4 formulation was considered an optimized nanoparticulate formulation.

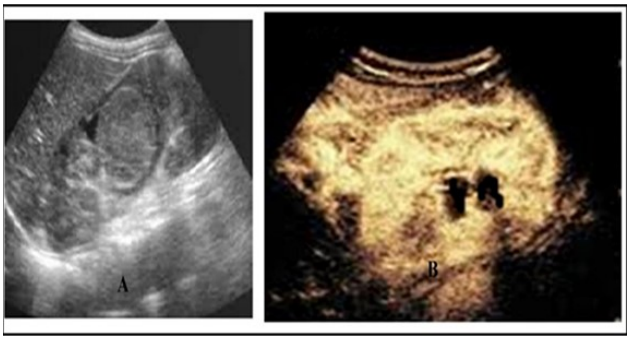

Fig. 1: A: Conventional Ultrasound imaging before injection of CRMN nanoparticles to animals; B: color doppler imaging before injection of CRMN nanoparticles to animals.

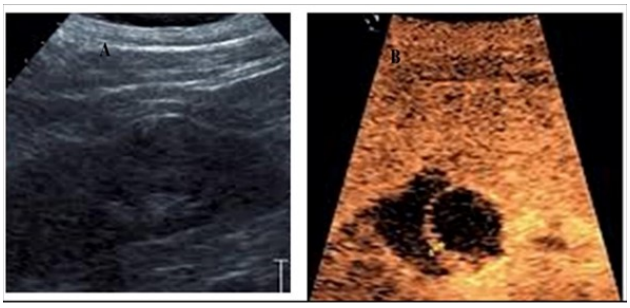

Fig. 2: A: Conventional Ultrasound imaging after injection of glycerin to animals; B: CEUS imaging after injection of glycerin to animals.

Evaluation of clinical parameters

The various clinical parameters like serum creatinine, serum urea nitrogen, resistance index, and peak systolic velocity were determined before injection, at 8 hours of injection, and after 24 hours of injection and parameters were compared. As can be seen from table 3 clinical parameters were found significantly changed when compared with before the injection of CRMN-loaded nanoparticles. The results were found to be statistically significant ($p<0.05$). These changes in values demonstrated the effect of CRMN-loaded nanoparticles on ovarian cancer. The Observations found in color Doppler examinations were supported by the changes in clinical parameters due to the use of CRMN-loaded nanoparticles (Liu *et al*., 2012; Wang *et al*., 2021).

Color Doppler examination

In this research work, we have developed an OC model in rabbits, and the diagnosis was carried out by the color Doppler technique. The application of the color Doppler technique for the study of various organs including liver, kidney and brain has been done in many animal models. The color doppler technique is considered one of the advanced techniques that makes easy real-time observation of vascular perfusions and observation of other organs (*Samir et al.,* 2021). The images before and after injection of CRMN-loaded nanoparticles were captured and before induction of OC all biological parts of the ovary were found to be normal and these are more distinct observed in images captured by color doppler technique as compared to conventional ultrasound technique. In color doppler, we also observed continuous and slow enhancement in ovarian arteries and other cellular and tissue vasculature. While conventional ultrasound technique didn't show any microvascular changes caused due to the injection of CRMN nanoparticles. The color doppler images demonstrated superiority in terms of imaging clarity and efficiency as compared to conventional ultrasound imaging technology. The OC changes are visible in the case of color doppler images which would be beneficial for the diagnosis and proper treatment. Collectively, these images of color doppler in animal model demonstrated efficient use in the diagnosis of micro vascular perfusion under different settings.

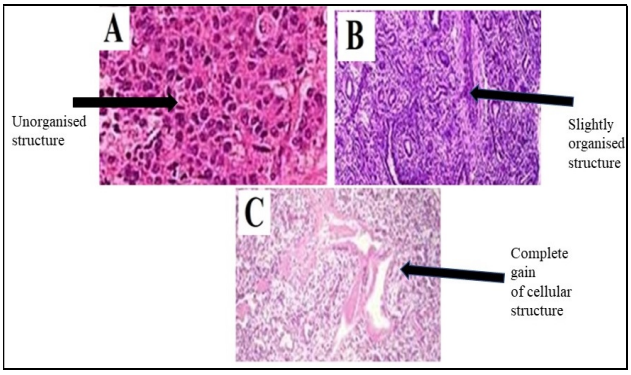

Fig. 3: Histopathological analysis of ovary: A) Before injection of CRMN nanoparticles; B) after 6 hrs. of injection and C) after 24 hrs. of injection.

Histopathological analysis

To support the observations found in color doppler examinations and changes in clinical parameters histopathological analysis was conducted on the cancerous ovary. The analysis was performed before injection, after 8 hrs and 24 hrs. after injection. As can be seen from fig. 3 significant changes have been observed after the injection of glycerin. Fig. 3A shows the

abnormal biological appearance of the ovary with a loss of organized structure. But after injection at 6 hours of time point slightly organized structure was observed. The scenario became very excellent at 24 hours of injection with a complete gain of cellular structure.

The aforementioned investigation is directed toward the development of curcumin loaded nanoparticles for efficient treatment of ovarian cancer which was further investigated by the color doppler ultrasound technique. The study will propose a new drug delivery technique in the form of nanoparticles to treat ovarian cancer more effectively.

CONCLUSION

The development of curcumin loaded chitosan nanoparticles were found to be useful in the treatment of ovarian cancer which was supported through the observation of the color Doppler technique. We successfully developed an OC animal model in this investigation, and the alterations were seen using color doppler imaging, as well as changes in clinical parameters and histology. The potential of curcumin-loaded nanoparticles in ovarian cancer was confirmed in this study, which used a gynecological color Doppler ultrasonography technology.

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