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Comparative study of radiolabeled 68Ga DOTANOC acetate, 68Ga PSMA-11 and 68Ga RGD on DEN and Phenobarbitone induced hepatocellualar carcinoma in Sprague dawley rats

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ABSTRACT
Liver cancer the third most commonly diagnosed cancer and fourth most common cause of mortality. The survival rate can be increased by earlier diagnosis and better treatment regimen. PET/CT has recently become a vital part to achieve this goal by providing the detailed information regarding tumor lesions than other techniques. Combination of FDG PET/CT has been used initially and proven to be of great benefit for the assessment of liver cancer. Due to some limitation of FDG, a new diagnostic tracer has to be introduced. Radiolabelled peptides are widely used now a days for imaging cancer lesions. The present study aimed to compare the three radiolabelled peptides ^{68}Ga DOTA-NOC acetate, ^{68}Ga PSMA-11 and ^{68}Ga RGD efficiency on DEN and Phenobarbitone induced hepatocellular carcinoma in Sprague dawley rats by PET/CT imaging. Radiolabeling efficiency of ^{68}Ga with DOTANOC, PSMA, RGD peptides were evalauated and found to be good. PET/CT imaging were carried using three peptides in which ^{68}Ga PSMA-11and^{68}Ga DOTA-NOCalone shows positive imaging. Other peptide acetate and ^{68}Ga RGD were not able to image the tumor lesions. Plasma clearance were also evaluated and found higher tumor to background ratio and a low accumulation in non-target organs for ^{68}Ga PSMA and ^{68}Ga DOTA-NOC. Thus on comparison, ^{68}Ga PSMA-11 and ^{68}Ga DOTA-NOCpeptide ismore suitable for preclinical imaging of colon cancers.
KEY WORDS:PET/CT, ^{68}Ga DOTA-NOC acetate,^{68}Ga PSMA-11,^{68}Ga RGD, liver cancer

1. INTRODUCTION

Cancer is a disorder that results from genetic or epigenetic changes which ultimately drive the malignant transformation of the normal cells. Cancer is most deadly and a threat to the developed and developing nation and is the second most common cause of death. By 2020, the world population is expected to have increased to 7.5 billion; of this number, approximately 15 million new cancer cases will be diagnosed, and 12 million cancer patients will die.[1]

The fourth-most common cancer in the world, liver cancer is characterized by abnormal cell growth in the liver .Liver cancer is much more common in countries in sub-Saharan Africa and Southeast Asia than in the US. In many of these countries it is the most common type of cancer. More than 800,000 people are diagnosed with this cancer each year throughout the world. Liver cancer is also a leading cause of cancer deaths worldwide, accounting for more than 700,000 deaths each year.[2] Many diagnostic methods are there to diagnose liver cancer. PET/CT imaging is the most advanced technique which helps to identify the location of tumor and stage of tumor. For PET/CT imaging new diagnostic tracer are developing for accurate imaging. Radiolabeled peptides are currently trending for imaging cancer as it binds to specific receptors in cancer cells due to overexpression of receptors, thus provides proper imaging.[3]

In the developing world, viral hepatitis (primarily hepatitis B), continues to represent the major risk for the development of HCC. The impact of hepatitis B vaccination on the eventual rate of HCC remains to be determined. [14] The results of the vaccination of newborns are encouraging.Other trends driving the epidemic include the aging population, obesity, and, perhaps, improved survival of patients with cirrhosis through better management of ascites and portal hypertension. The worldwide burden of HCC is also likely to continue. While significant progress has been made worldwide through HBV vaccination as part of the expanded program for

vaccination by the World Health Organization (WHO), the prevalence of chronic liver disease remains significant among the older population who is at risk of developing HCC.

In recent years, many radiolabeled peptide analogs, such as somatostatin, bombesin, vasoactive intestinal peptide, cholecysto-kinin/gastrin, neurotensin, exendin and RGD derivatives, have been developed for scintigraphic detection of different tumor types.[22]Several studies found that expression of receptors and transporters are high in density during carcinogensis phase of liver cancer. The purpose of the study is to comparing various radiolabeled peptides like ^{68}Ga DOTANOC acetate, ^{68}Ga PSMA-11, ^{68}Ga RGD expression in liver cancer cells.

DEN Diethyl nitrosamine solution is freshly prepared before the induction. DEN is dissolved in 1 mMol/L EDTA-normal saline, the pH is adjusted to 6.5 with 1 Mol/L NaOH to ensure the pH suitability and the stability of the chemical.[60]

2. METHODOLOGY

2.1 INDUCTION OF LIVER CANCER:

DEN is being administered intra peritoneallydose of 200mg/kg body weight at single inductionafter 2 nd week phenobarbitone given orally with drinking water till 16 weeks.

2.2 EXPERIMENTAL DESIGN:

Rats were randomly divided into five groups, 6 animals each, placed in individual cages

Group (1): Control Group: Rats were treated with only 0.9%w/v normal saline daily throughout the experiment.

Group II :(DEN induced group):DEN is being administered intra peritoneallydose of 200mg/kg body weight at single inductionafter 2 nd week phenobarbitone given orally with drinking water till 16 weeks.

Group III :(DEN+ ^{68}Ga DOTA NOC):DEN is being administered intra peritoneallydose of 200mg/kg body weight at single inductionafter 2 nd week phenobarbitone given orally with drinking water till 16 weeks followed by administration of ^{68}Ga DOTA-NOC acetate intravenously to the rats at the day of examination.

Group IV :(DEN+ ^{68}Ga PSMA-11):DEN is being administered intra peritoneallydose of 200mg/kg body weight at single inductionafter 2 nd week phenobarbitone given orally with drinking water till 16 weeks followed by administration of ^{68}Ga PSMA-11 intravenously to the rats at the day of examination.

Group V :(DEN+ ^{68}Ga RGD):DEN is being administered intra peritoneallydose of 200mg/kg body weight at single inductionafter 2 nd week phenobarbitone given orally with drinking water till 16 weeks followed by administration of ^{68}Ga RGD intravenously to the rats at the day of examination.

2.3. Procedure for radioimaging:

2.4. Preparation of ^{68}Gallium:

^{68}Ga was eluted from a commercially available ^{68}Ge/^{68}Ga generator with 4 ml of 0.05MHCl.The pH was adjusted to 4- 4.5 by adding sodium acetate to the preparation.[75]

2.5. Radiolabeling of peptides:

Allowed the peptide kit vials (DOTA-NOC, PSMA-11, RGD) to attain room temperature. 20mcg of DOTANOC acetate, 40 mcg of PSMA-11 and 50mcg of RGD was added separately to each gallium vials and gently mix it. Incubated the vials at 90^{O} C in a water bath for 10 mins and allowed to cool for few minutes and carry out the quality control test.[76]

3.QUALITY CONTROL TEST:

3.1Instant Thin Layer Chromatography (ITLC):

Radiolabeling efficiency (%) of the radiolabeled conjugates was determined by ITLC using silica gel (SG) impregnated sheets. 1.0 M ammonium acetate (NH4OAc) was prepared by dissolving 770.0 mg of NH4OAc in 10.0 mL of distilled water. The ITLC sheets were cut into strips of dimensions 11.0 cm X 0.5 cm. The ITLC strips were marked 1.0cm above the base as origin. 2.0 µL of the free 68Ga and radiolabeled preparation was spotted at the origin, allowed to dry and placed in test tubes preloaded with mobile phase i.e.1.0 M ammonium acetate:methanol (1:1). The mobile solvent was allowed to reach the demarcation at the top of ITLC strip. The strip was taken out, dried and radioactivity counts over the entire strip were recorded using a radio chromatographic scanner. Further, the ITLC strips were also cut in two halves, the lower one-third and the upper two-third in order to calculate the radiolabeling efficiency. Both the segments were measured for radioactivity in a well counter. The counts from lower one-third segment of ITLC strip depicted (%) free 68Ga whereas the (%) 68Ga labeled DOTANOC acetate, PSMA-11, RGD was calculated from the upper twothird segment of the ITLC strip.[77]

3.2ADMINISTRATION OF RADIOLABELED PEPTIDES:

PET/CT scans were obtained with a PET/CT Scanner (Siemens Medical Solutions). Rats were each injected via the tail vein with 100µCi of the [68Ga]DOTANOC acetate, PSMA-11, RGD respectively under ketamine and xylazine anesthesia.After the injection of radiolabeled compounds, rats were subjected to PET/CT

imaging at an interval of 15, 30, 60, 90 minutes under anaesthetic condition.For attenuation correction and anatomical reference, CT images were acquired following PET imaging. All animals were visually monitored throughout the imaging procedure.

3.3 COLLECTION OF BLOOD FOR PHARMACOKINETIC PARAMETERS

After the PET/CT imaging, the blood was collected from Retro-orbital sinus by using capillary tube into a centrifugation tube which contains EDTA for pharmacokinetic parameters. Plasma was separated by centrifugation at 10000 rpm for 10 min and utilized for pharmacokinetic parameters like plasma clearance wereestimated.

3.4 PREPARATION OF WELL COUNTER SAMPLES

Weighed samples of 500 µL ofplasma were prepared in duplicate. A volume of 50 µL 10% Triton-X (ICN Biomedicals Inc.) was added to the samples to destroy blood cells and obtain homogeneous solutions for measurement and avoid geometric effects caused by the formation of blood cell pellets.[78]

3.5 DETERMINATION OF PLASMA CLEARANCE:

Weighing method is used, transfer the syringe contents (standard) into a volumetric flask (500ml) the syringe must not be rinsed. Fill the volumetric flask with water to the 500-ml level and mix the solution thoroughly. Pipette twice 1.0ml of this new solution into counting vials. Substantial variations in counts between standard samples indicate an error, either in pipetting or in the homogeneity of the solution. New samples from the dilution of the standard should then be prepared. After selection of the adequate energy peak and window, both the blood samples and the standard are measured in a well counter. For quality control reasons, each plasma sample and standard vial should be counted twice. A background activity should be measured in the beginning and at the end of the counting.[79]

3.6 HISTOLOGICAL ASSESSMENT[60]

Histopathology is the microscopical study of tissues for pathological alterations. This involves the collection of morbid tissues from biopsy or necropsy, fixation, preparation of sections, staining and microscopical examination.

4 RESULTS

4.1 Instant thin layer chromatography (ITLC)

Radiolabeling efficiency (%) of the final radiolabeled product [68Ga] DOTANOC acetate, PSMA-11, RGD was estimated by using ITLC technique.The computer generated chromatogram depicting the movement of free 68Ga and [68Ga] DOTANOC acetate, PSMA-11, RGD along the ITLC strip as a function of radioactivity counts are presented in Figure (1),(2),(3) and (4)respectively.The results indicated that radiolabeling efficiency of DOTANOC acetate as 85.78%, PSMA-11 as 48.74%, RGD as 68.44% was achieved at pH 4.0.

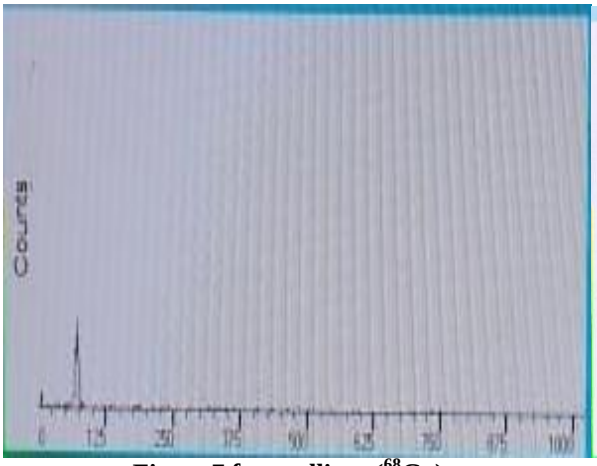


Fig no 7.free gallium (^{68}Ga)

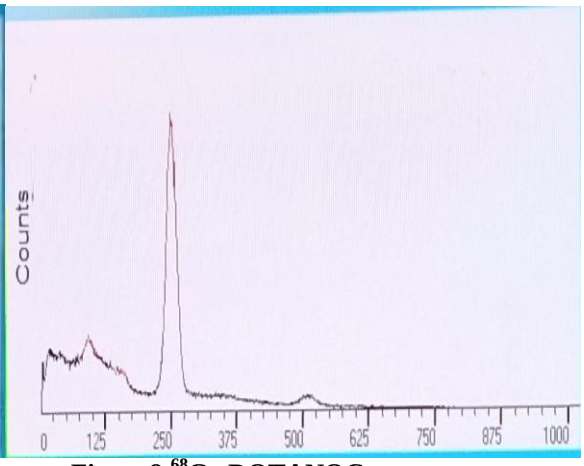


Fig no 8.^{68}Ga DOTANOC acetate

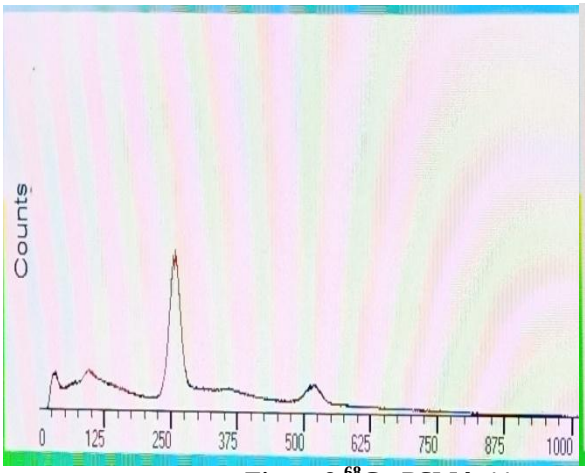


Fig no 9.^{68}Ga PSMA-11

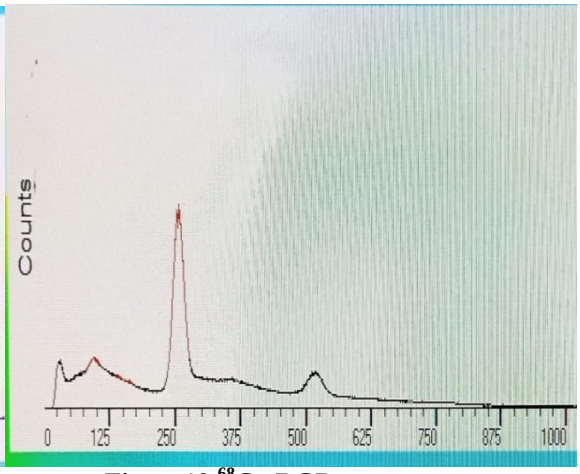


Fig no 10.^{68}Ga RGD

4.2 PET/CT IMAGE:

GROUP I : Normal

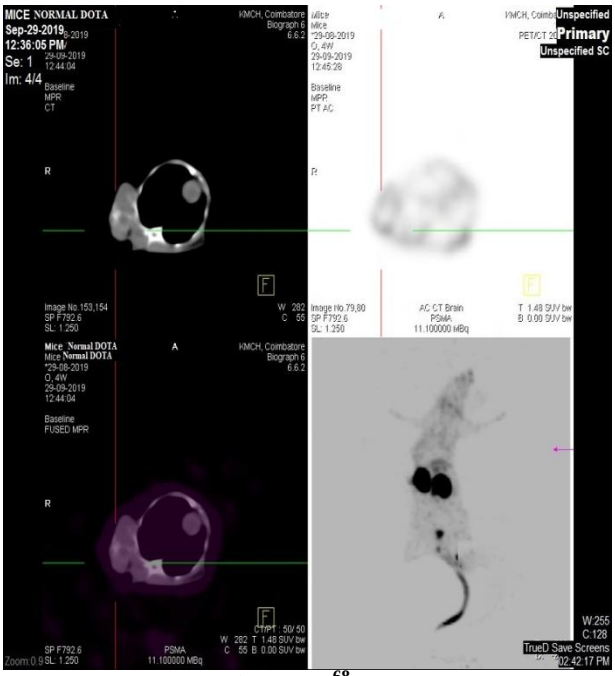


Fig no 11.^{68}Ga DOTANOC acetate

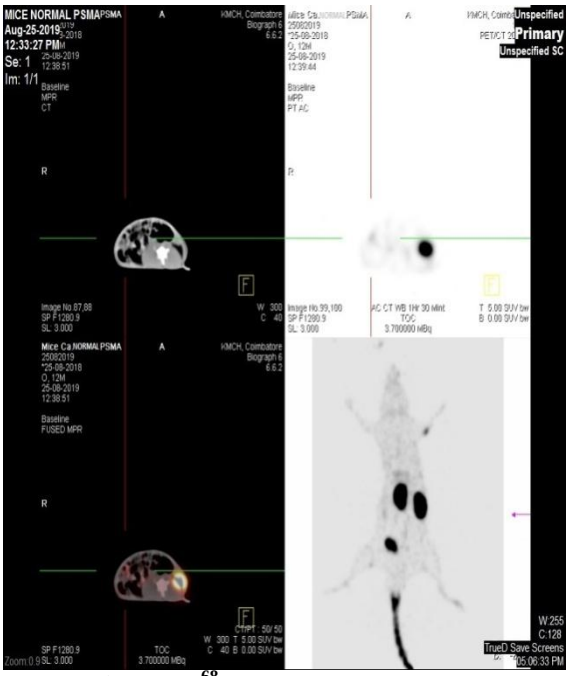


Fig no 12.^{68}Ga PSMA-11

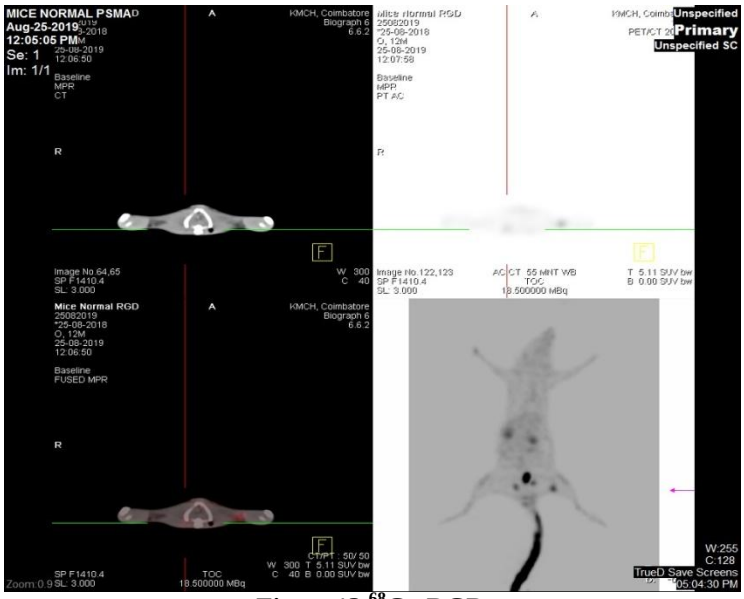
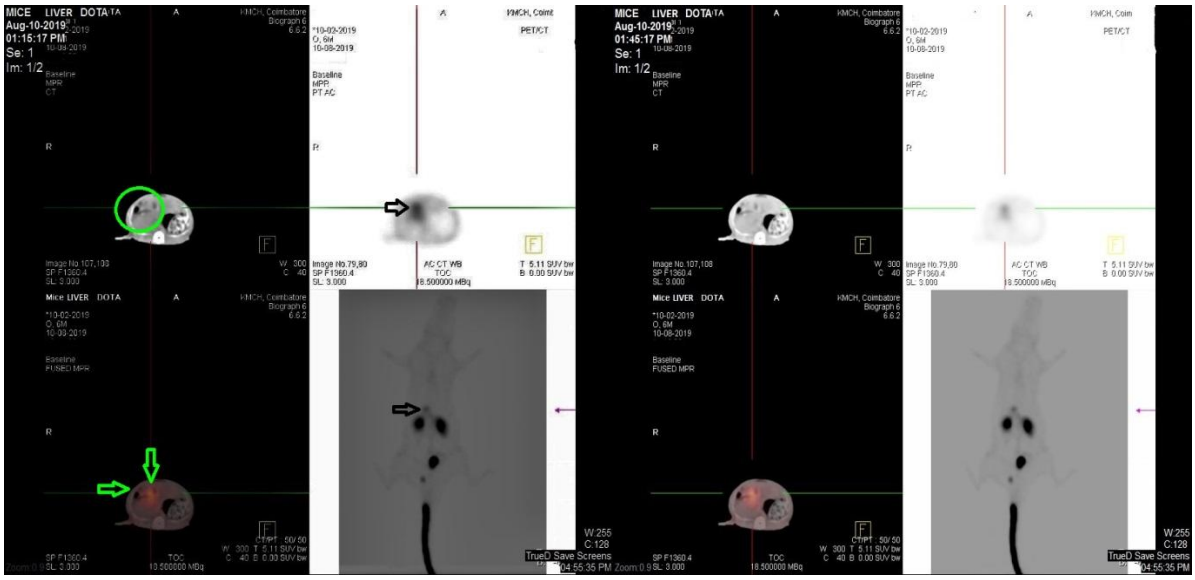
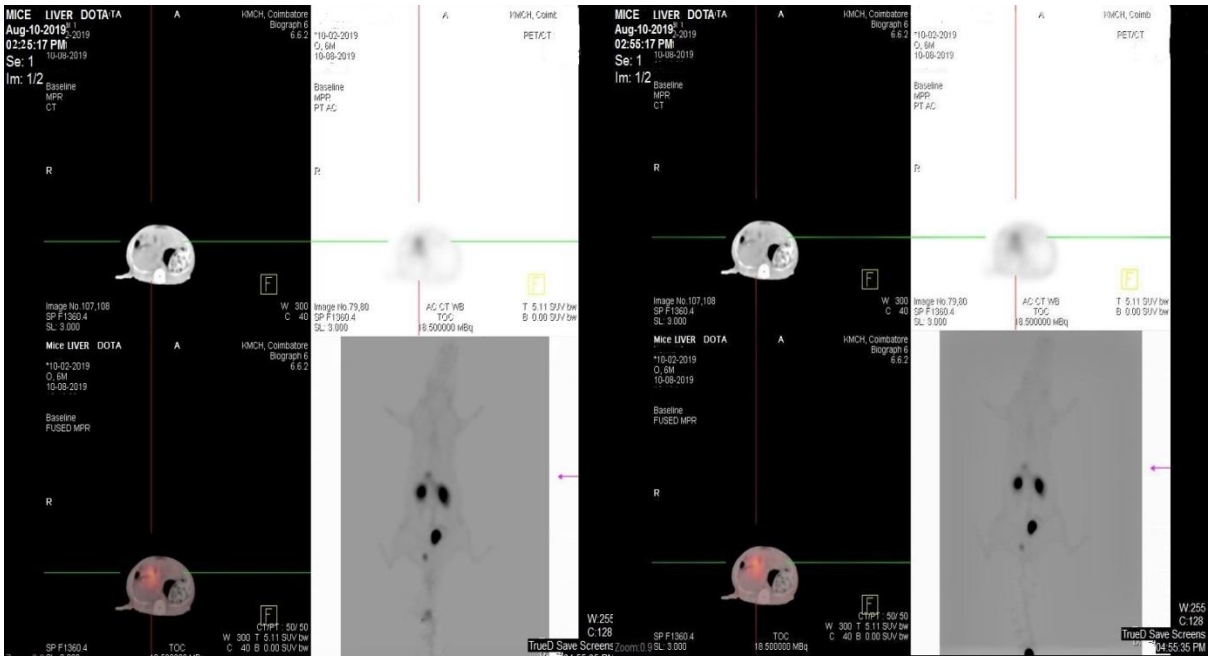


Fig no 13.^{68}Ga RGD

GROUP- III: ^{68}GaDOTANOC acetate



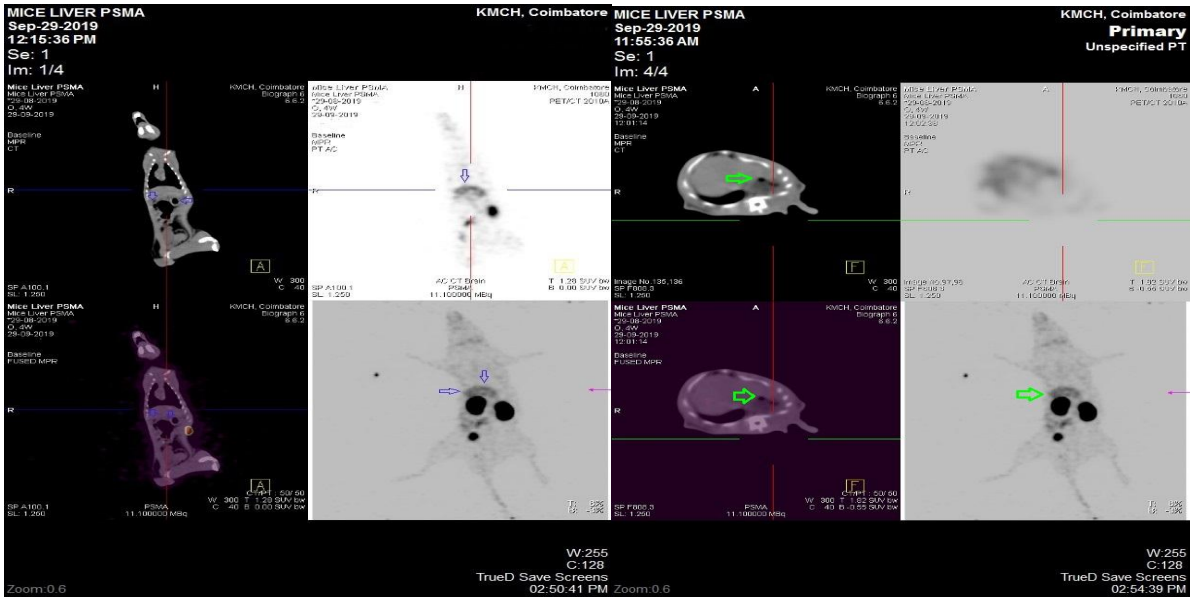
a) 15 Mins b) 30 Mins



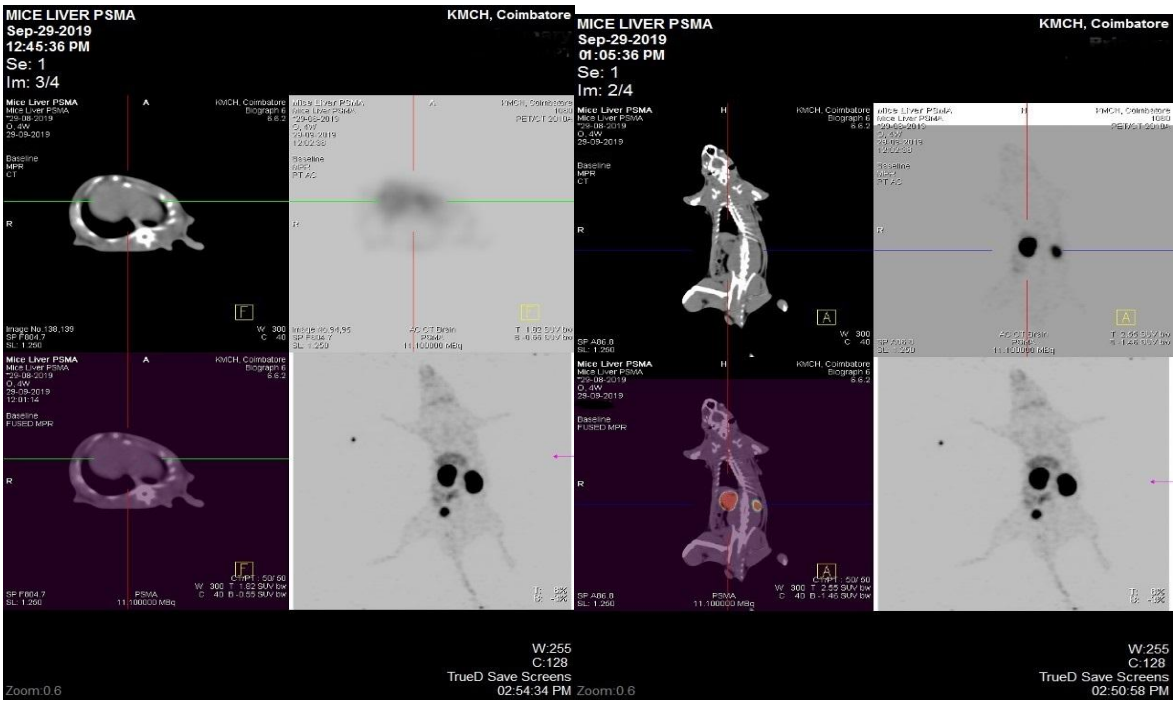
c) 60 Mins d) 90 Mins

Fig no 14. Imaging of rat with ^{68}Ga DOTANOC acetate

GROUP IV:^{68}Ga PSMA-11



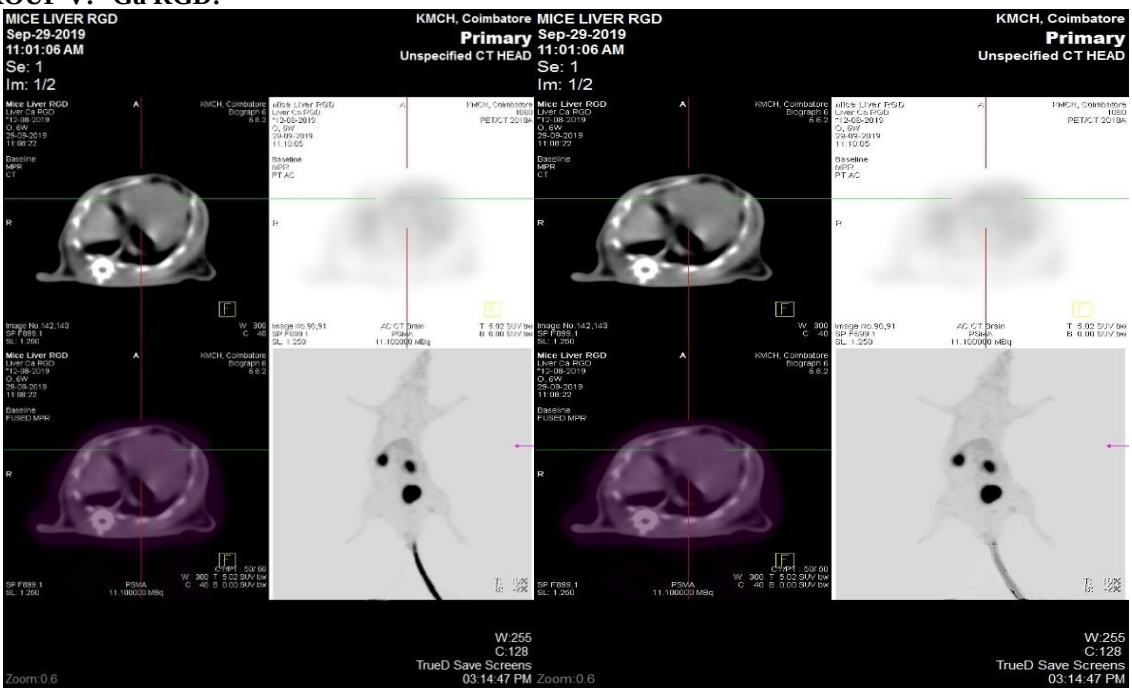
a) 15 Mins b) 30 Mins



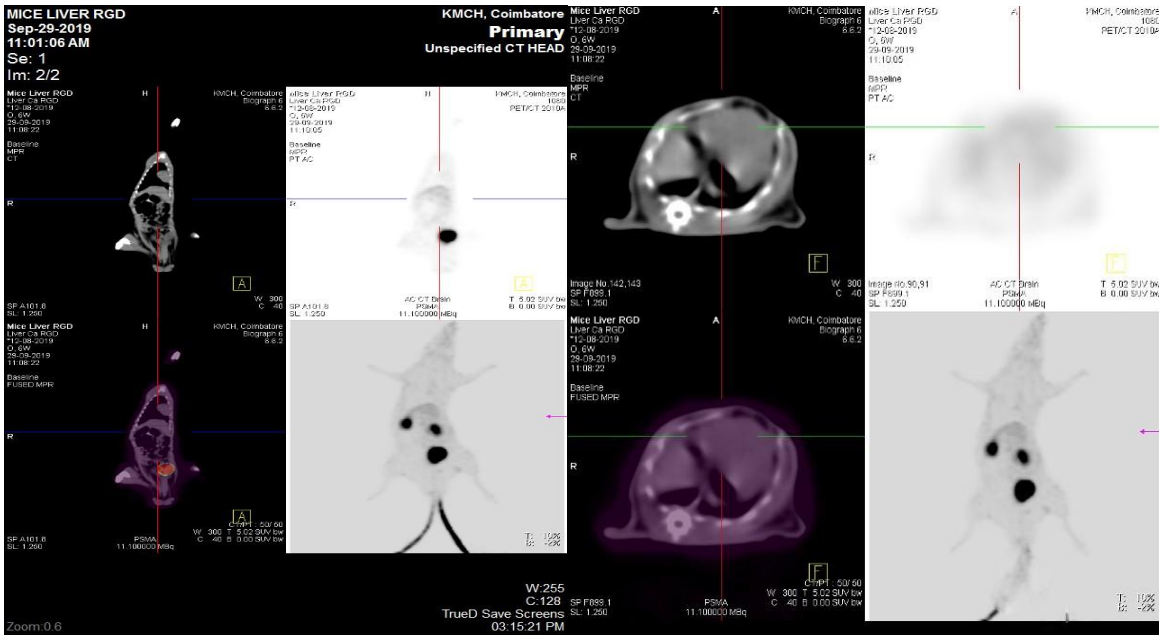
C) 60 Mins d) 90 Mins

Fig no 15. Imaging of rat with ^{68}Ga PSMA-11

GROUP V:^{68}Ga RGD:



a) 15 Mins b) 30 Mins



C) 60 Mins d) 90 Mins

Fig no 16. Imaging of rat with ^{68}Ga RGD

4.3 GROSS NECROPSY STUDY

After PET/CT imaging animals were fasted overnight, anaesthetised and the abdomen was cut open to remove the liver to investigate the morphology, pathology (gross necropsy study). The results of the finding were depicted in Figure no: 14 and 15

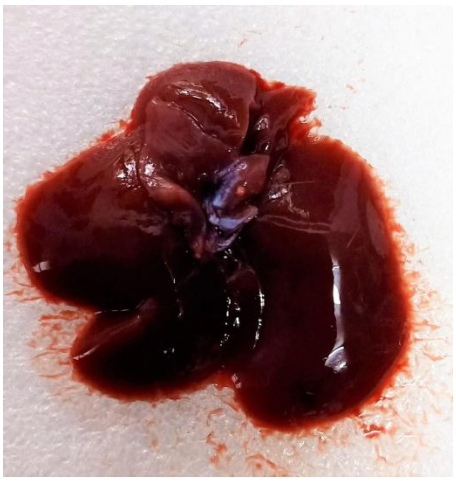


Fig no 17. GROUP – I CONTROL

Animals in group - I which were treated only with normal saline showed no changes in the liver.

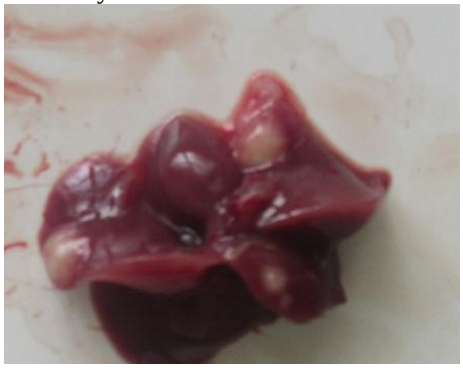


Fig no 18. GROUP – II Only DEN and phenobarbitone (200mg/kg)

Animals in group - II which were treated with DEN and Phenobarbitone induced liver cancer showed changes like polyps formation when compared to control group

4.4 PHARMACOKINETIC EVALUATION

Blood samples were withdrawn at 15 min, 30 min, 60 min and 90 min following the intravenous administration of the radiolabeled peptides (^{68}Ga DOTANOC acetate, ^{68}Ga PSMA-11, ^{68}Ga RGD) in the rats. The blood counting of the radioactivity was evaluated by using well counter. Following table shows the counts of radiolabeled peptides at different intervals.

Time (Mins)	Counts					
	^{68}Ga DOTANOC acetate		^{68}Ga PSMA-11		^{68}Ga RGD	
	Group 1	Group 3	Group 1	Group 4	Group 1	Group 5
15	46338	49567	35208	37508	40228	41258
30	45997	44012	34726	32568	39894	40745
60	44117	38829	33991	29654	39016	39973
90	43017	33456	33007	25618	38456	39029

Table no:4 Plasma clearance counts of radiolabeled peptides

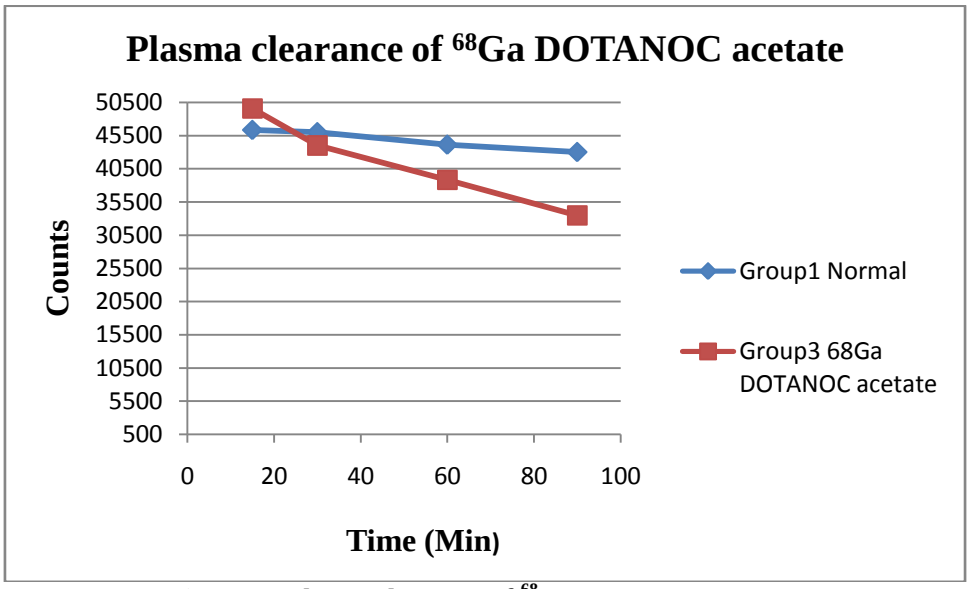


Fig no 19. Plasma clearance of ^{68}Ga DOTANOC acetate

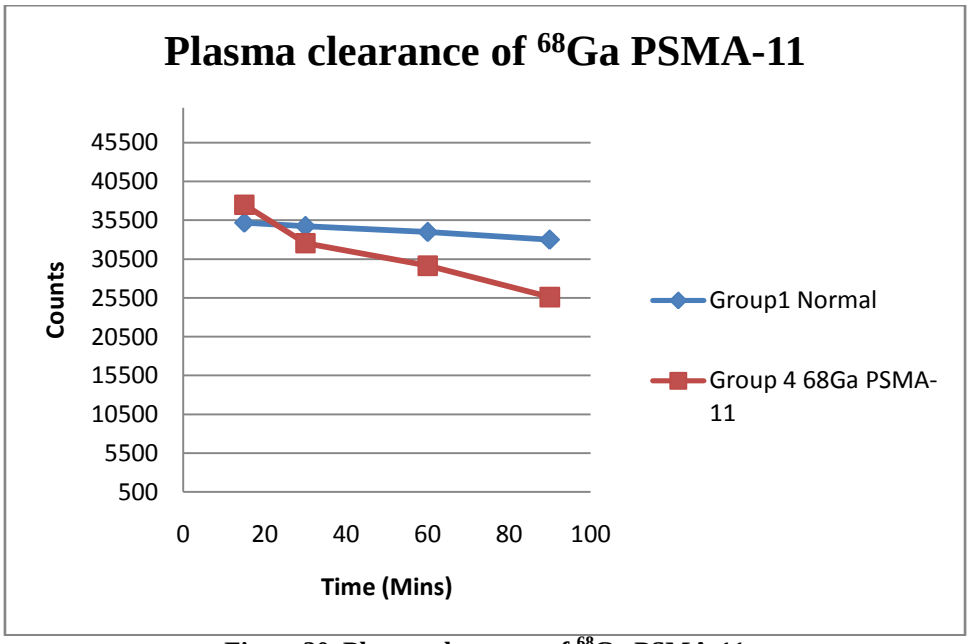


Fig no 20. Plasma clearance of ^{68}Ga PSMA-11

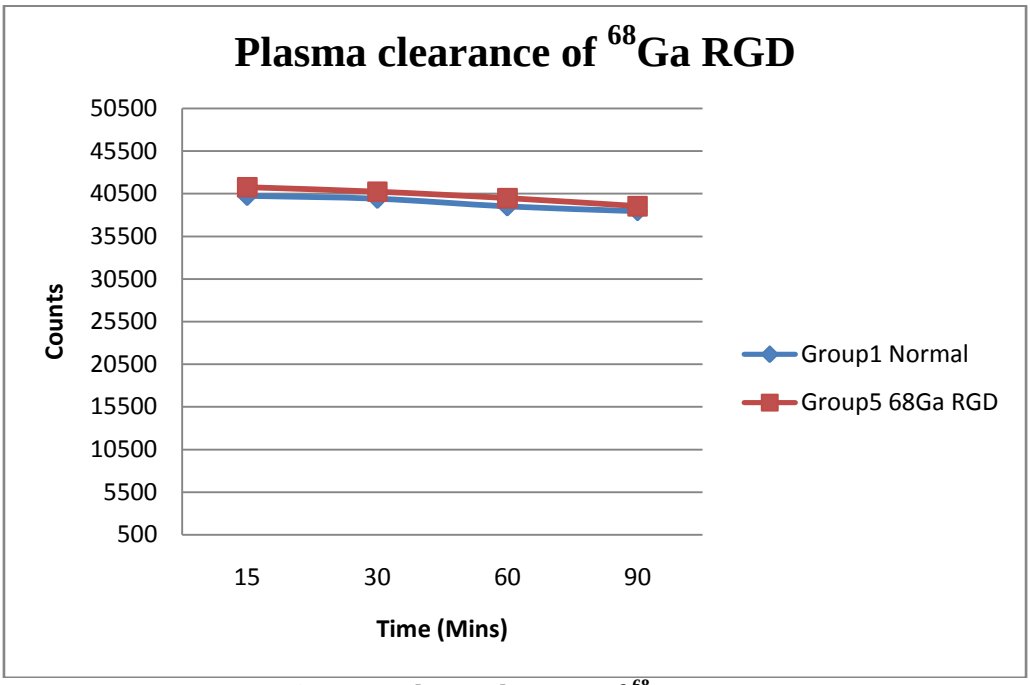
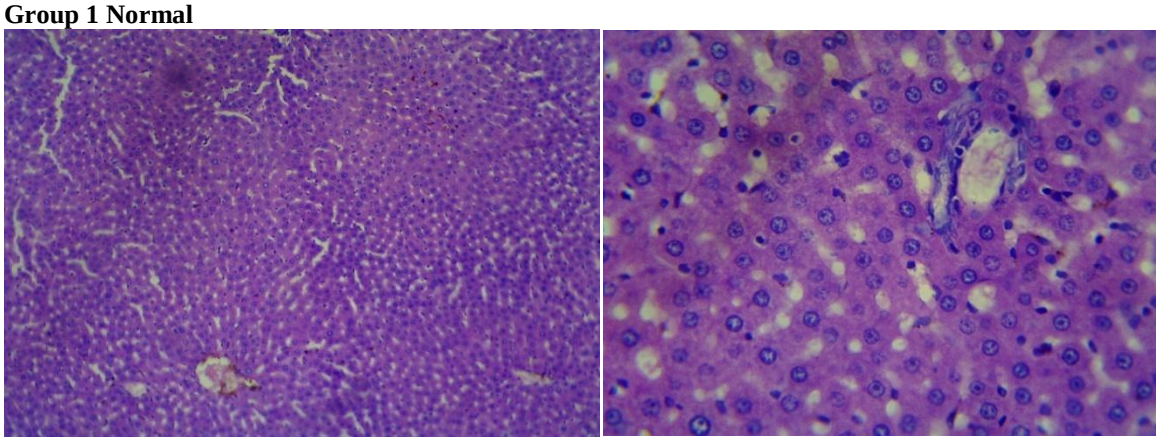


Fig no 21. Plasma clearance of ^{68}Ga RGD

4.5 HISTOPATHOLOGY:

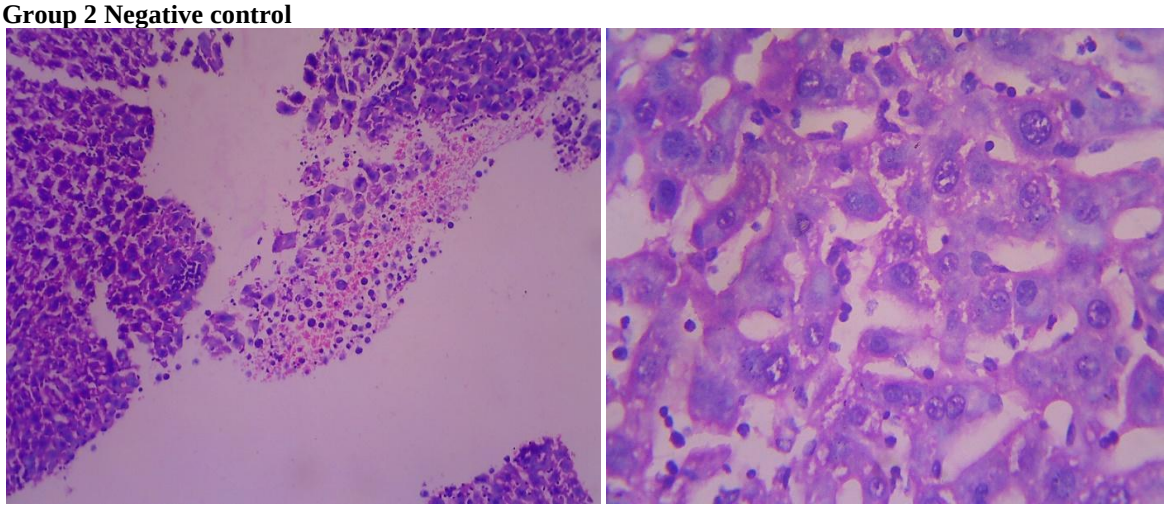
Group 1 Normal



10X **40X**

Section studied from the liver shows normal lobular architecture. The sinusoids show normal. The portal tract shows normal morphology. The central vein shows mild dilatation. There is no lobular inflammation, necrosis or granulomas in the liver parenchyma.

Group 2 Negative control



10X **40X**

Section from liver shows dysplastic changes.shows tumor cells are round to oval having moderate eosinophilic cytoplasm and round to oval vesicular nuclei with a prominent nucleoli. Surrounding stroma shows tumor cells. .Bile duct shows hyperplasia.

5. DISCUSSION

PET one of the molecular imaging technology are widely used in medicine to provide information regarding pharmacokinetic parameters and efficiency to reach the target site by new molecular entity which might helpful for new drug development for both therapeutic and diagnostic purposes. In case of oncology it has essential role to detect the various stages of disease progression and also to identify how well the therapy reacts with disease so that physician can decide about future treatment regimens.[80]

A variety of imaging probes have been developed for different molecular targets. Initially flurodeoxyglucose has been developed based on the principle that cancerous cells uptake glucose in high concentration than the normal cells. But targeting by this probe is not sufficient to decide the further therapy for clinical patients. Hence peptide based targeting probes has been introduced, as receptors expression will be higher on cancerous cells than normal. This makes peptide to target on the particular receptor in higher density thereby makes better diagnosing ability.[81]

DOTANOC an octreotide analogue acts on the somatostatin receptor specifically somatostatin receptor type 2, 3, 5. The peptide is initially developed for neuroendocrine tumors localization and internal radionuclide therapy. It is found that somatostatin receptors are over expressed in several human tumors especially neuroendocrine tumors and their metastases these tumors can visualized invivo by radiometal labeled peptides.

Many studies has been done to evaluate the efficiency of this peptides for both diagnosing and radiotherapy characteristics.[82]

PSMA-11 targets the transmembrane glycoprotein prostate-specific membrane antigen (PSMA). PSMA is widely expressed in normal epithelium of the prostate gland and to a lesser extent in other tissues such as the brain, salivary glands, intestines, liver and kidney. Its expression is high in benign as well as in malignant prostate tissue. PSMA is also present in the neovasculature of solid tumors including kidney, lung, stomach, colon, and breast. Based on the antigen antibody reaction, it targets specifically hence consider as better diagnostic imaging.[62]

RGD (arginine glycine aspartate) peptide has a high affinity toward αvβ3 integrin receptors It is well documented that integrin αvβ3 plays an important role in the regulation of tumor growth, angiogenesis, local invasiveness, and metastatic potential. Integrin αvβ3 is upregulated on the activated tumor endothelial cells and also highly expressed on some tumor cells such as glioblastoma, breast and prostate tumors, malignant melanomas, and ovarian carcinomas. Radiolabeled RGD (Arg-Gly-Asp) peptides and analogs that specifically target integrin αvβ3 have been widely tested for tumor imaging in pre-clinical and clinical studies.[83]

Several PET radionuclides has been introduced, amongst all ^{68}Ga has renewed interest due to its several benefits. Firstly, it has a high accessibility for users and is economically advantageous because a cyclotron is not required. Secondly, ^{68}Ga- based radiopharmaceuticals produce high spatial resolution compared with single-photon emission computed tomography (SPECT), allowing more accurate quantification. Lastly, shorter half-life of 68 min.[71]

In this study the efficiency of three radiolabelled peptides (^{68}Ga DOTANOC acetate, ^{68}Ga PSMA-11 and ^{68}Ga-RGD,) on hepatocellular carcinoma are compared under preclinical settings by PET/CT scan. The peptides are labelled with radionuclide ^{68}Ga and radiolabelling efficiency were identifed by ITLC. The ITLC paper were characterized using well counter and found the radiolabeling efficiency of DOTANOC acetate as 85.78%, PSMA-11 as 48.74%, RGD as 68.44% at pH 4.0 from the upper two third segment of the paper. The lower segment shows 2-3% of radioactivity due to free gallium. Hence the radionuclide has been well binded to the peptides.

From PET/CT imaging it is shown that ^{68}Ga PSMA and ^{68}Ga DOTANOC acetate alone reaches the liver cancer tissues whereas peptide ^{68}Ga-RGD shown no activity around the colon tissues. Apart from liver tissues ^{68}Ga-tracers of all the peptides showed kidneys and bladder as the organs with the highest accumulation of radioactivity. It is noted from the imaging that expression of PSMA might be high in the region of colon cancer tissue hence the ^{68}Ga PSMA and ^{68}Ga DOTANOC acetate has a good diagnostic property for liver cancer apart from prostate cancer and neuroendocrine tumor.

Plasma clearance were estimated by well counter method for all the three radiolabeled peptides. The counts of radioactivity of peptides in the cancer induced groups were compared with normal group. It is found that all the three peptides injected in the normal group rats showed high radioactivity in plasma after 15 minutes of intravenous injection which is slightly decreases after 30, 60, 90 mins. ^{68}Ga PSMA-11 and ^{68}Ga DOTANOC showed gradual decrease in the each intervals as compared to normal group rats which might be because of the uptake of ^{68}Ga PSMA-11 and ^{68}Ga DOTANOC by cancer cells. The other peptide radiolabeled peptides decrease in their radioactivity in plasma, but resembles similar to the normal group counts. Hence the uptake of and ^{68}Ga-RGD by tissues were less.

6. CONCLUSION

The current study showed the tumor imaging capacity of ^{68}Ga DOTANOC acetate, ^{68}Ga PSMA 11 and^{68}Ga-RGD peptides on DEN and phenobarbitone induced hepatocellular carcinoma in spraguedawley rats. Among the three radiolabelled peptides ^{68}Ga PSMA 11 and DOTA -NOC showed excellent imaging capacity; on the contrary other peptide^{68}Ga RGDwere not able to image the tumor might be because of low penetration of these peptides in HCC tissues. ^{68}Ga-PSMA 11 peptides also showed higher tumor to background ratio and a low accumulation in non-target organs except the excretory organs compared to DOTA- NOC imaging capacity. From these properties make ^{68}Ga-PSMA-11and DOTA- NOC peptidesmore suitable for preclinical imaging of hepatocellular carcinoma cancers.

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