Chapter VII

Tumor control by chemo-targeting of Nanoparticle-drug complexes: Control of solid tumor in mice by targeting Iron-oxide nanoparticle – Berberine complexes with Sanazole

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Tumor hypoxia - associated resistance to radiation and chemotherapeutics is a major problem in the treatment of malignant solid tumors. The present study exploits tumor hypoxia, for specific targeting iron-oxide nanoparticle (NP) and cytotoxic drug Berberine (BBN) complexes conjugated with hypoxic cell sensitizer Sanazole (SAN), to hypoxic solid tumors. Tumor regression and the underlying mechanisms were studied following the administration of NPs, drugs and NP-drug complexes. Tumor markers, serum parameters, antioxidants, histopathology and survival of the tumor-bearing animals were also investigated. Tumor volume was found to be significantly reduced in NP-BBN-SAN treated group compared to the control and other groups (treated with NP, BBN, SAN, NP-BBN and NP-SAN). Extensive damage to cellular DNA and down regulated expression of *hif-1α*, *vegf* and *akt* - genes involved in hypoxic acclimatization - were observed in NP-BBN-SAN group in comparison with the control. The up regulation in the expression of genes for extrinsicand intrinsic pathway of apoptosis indicated the involvement of both in tumor regression. The results from tumor markers, antioxidant-status and serum parameters were corroborated the molecular findings. Tumor specific action of these complexes could be realized from the histopathological data on tumor, liver and kidney tissues. The animals administered with NP-BBN-SAN complexes showed 100% survival and no reoccurence of tumor were noticed for 30days. Hypoxia specific chemodirected targeting (with SAN) of NP-BBN complexes enhanced the therapeutic efficacy of the cytotoxic drug berberine. The use of nanoparticles and sanazole could be an effective method of specific targeting of cytotoxic drugs to hypoxic cells in solid tumors.

7.1 INTRODUCTION

The success of chemotherapy is often limited by systemic toxicities and side effects. One of the means of overcoming these problems of antineopalstic agents is to deliver the drugs at the tumor site [Bae and Park, 2011].Hypoxic region in tumor supports not only tumor growth but also resistance to major therapies such as chemo - and radiation - therapy [Cosse and Michiels, 2008; Rockwell et al., 2009].The major cellular adaptive response under the reduced oxygen state in tumor tissues is the expression of hypoxia-inducible factor-1 (HIF-1), especially HIF-1α.HIF-1 is a DNA- binding protein comprises heterodimer of O_2- regulated HIF-1α and a subsequently expressed HIF-1β, binds to hypoxia-responsive genes. Under normoxia, HIF-1α is degraded by ubiquitination while it is stabilized under hypoxia [Wang and Semenza, 1995; Wang et al., 1995].

The cells under hypoxic condition develop a series of adaptive responses to enhance tumor progression. Among these responses, altered expression of genes for angiogenesis and cell proliferation permit the cells to take more O_2. The changes in the expression of genes for metabolic pathways such as glycolysis are also reported in this condition. Most of these

changes are mediated by HIF-1α, results in the development of drug resistance[Semenza, 2013; Ghattass et al., 2013]. Hence,to overwhelm tumor hypoxia and thereby enhance chemotherapy response in solid tumors, new therapeutic approach is essential. Several hypoxia- targeted methods are studied to enhance the therapeutic effectiveness. The hyaluronic acid- coated mannan-conjugated MnO_2nanoparticles were found to be effective in down- regulating HIF-1α via increasing tumor oxygenation. The doxorubicin conjugated with these nanoparticles significantly increases the drug accumulation in tumor compared to chemotherapy with doxorubicin alone [Song et al., 2016]. Perche *et. al*. [Perche et al., 2016 and 2014] developed a hypoxia seeking copolymer forsiRNA deliveryto enhance tumor therapeutic efficacy.

Several hypoxic cell radiosensitizers have been developed [Shibamoto et al., 1987]. Shibamoto et al in 1986 reported that the various 3-nitrotriazole compounds especially Sanazole had noticeable radiosensitizing effects under both *in vitro* and *in vivo* conditions. The mechanism of sensitization by Sanazole is ascribed to increase in DNA damage [Pasupathy et al., 2001; Huilgol et al., 1996]. Sanazole was found to be effective in enhancing radiation-induced cellular damages and apoptosis in murine fibrosarcoma [Rajagopalan et al., 2003]. Das *et al* in 2004 demonstrated the potential application of sanazole in targeting hypoxic tumor in mice-bearing fibrosarcoma. By exploiting tumor hypoxia, we earlier reported that sanazole can specifically target iron oxide nanoparticle (NP)- doxorubicin complexes to tumor and thereby enhances the efficacy of the doxorubicin treatment[Sreeja and Nair, 2016].

The present study, the cytotoxic isoquinoline alkaloid Berberine (BBN) was delivered to tumor by conjugating with NP-SAN complexes. BBN is a component of several herbs used in Indian and Chineese traditional systems of medicine [Craig, 1999; Sathyavathi et al., 1987]. BBN has wide variety of therapeutic activities such as antibacterial [Kang et al., 2015; Peng et al., 2015], antiviral [Wu et al., 2011] and anti-inflammatory [Kuo et al., 2004].Recently, its anticancer properties were explored and found to be effective in reducing or inhibiting the growth of cancer cells by the activation of caspase-dependent apoptotic pathways [Choiet al., 2008; Ho et al., 2009; Hsu et al., 2007; Patil et al., 2010; Auyeung et al., 2009; Yu et al., 2007; James et al., 2011]. Apart from its apoptosis - dependent mechanism of cytotoxic action, it is found to be effective in arresting cell cycle as well as inhibiting cell migration and invasion via regulation of multiple pathways

[Singh et al., 2011; Li-Weber, 2013; Tsang et al., 2009; Zhang et al., 2010; Meeran et al., 2008; Pandey et al., 2008].

However, the major problem associated with berberine is its lack of hydrophilicity resulting in low effective concentration and poor bioavailability. To overcome this problem we used complexes of iron-oxide nanoparticles and hypoxic cell sensitizer sanazole in conjugation with berberine. This increased the therapeutic efficacy of BBN by enhancing bioavailability and specificity. We already reported that these complexes have potent cytotoxic activity against mouse lymphoma cells under *in vitro* condition [Sreeja and Nair, 2015b]. The purpose of the present study was to explore the use of these novel therapeutic strategy in the control of hypoxic tumor in an animal model.

7.2 MATERIALS AND METHODS

7.2.1 *Chemicals*

$FeCl_2$, $FeCl_3$, Chloroform and Isoamyl alcohol were bought from Merk, India. Berberine, chemicals for RNA isolation, β- D Glucuronidase, Brij and O- Dianisidine were purchased from Sigma Aldrich, India. Sanazole (AK2123) was obtained from Dr. V.T.Kagiya, Health Research Foundation, Kyoto, Japan. All other chemicals were obtained from reputed national manufactures (Otto Chemie Pvt. Ltd. and Himedia Pvt. Laboratories Ltd).

7.2.2 *Animals*

Female *Swiss albino* mice weighing 24-28g were purchased from the Small Animal Breeding Section (SABS), Government Veterinary College, Mannuthy, Thrissur, Kerala [Sreeja and Nair, 2015a]. They were kept under standard conditions of temperature and humidity in the Centre's Animal House Facility. The animals were provided with standard mouse chow (Sai Durga Feeds and Foods, Bangalore, India) and water *ad-libitum*. All animal experiments in this study were accomplished with the prior approval of the Institutional Animal Ethics Committee (IAEC) and were performed strictly adhering to the guidelines of CPCSEA constituted by the Animal Welfare Division of Government of India.

7.2.3 *Preparation and characterization of NPs and its complexes with drugs*

Co-precipitation method was used to prepare Fe_3O_4 nanoparticles.The nanoparticles thus formed were surface modified and complexed with the drugs - BBN and SAN (under

sonochemical process of 1:1:1 mixture of 0.2% solutions of NPs and drugs) - and characterized by IR- spectroscopy, XRD, TEM and size distribution analyses [described in chapter V].

7.2.4 *Tumor transplantation*

Dalton Lymphoma Ascites cells (5×10^6 in 0.1ml)in phospate buffered saline (PBS) were subcutaneously injected into the left hind limb of the animals. When the volume of the tumor reached approximately $1cm^3$, the NPs and NP-drug complexes (NP-SAN, NP-BBN and NP-BBN-SAN) were administered orally for twelve consecutive days in a similar manner as described in our previous study [Sreeja and Nair, 2015a].

7.2.5 *Experiment design*

The tumor-bearing animals were divided into seven groups of fourteen animals in each. Group 1: Control; tumor- bearing animals administered with PBS; Group 2: NP; tumor-bearing animals treated with NPs (20mg/kg); Group 3: BBN; tumor- bearing animals treated with BBN (1.34µmoles;20mg/kg); Group 4: SAN; tumor- bearing animals treated with SAN (2.18µmoles;20mg/kg); Group 5: NP-BBN; tumor- bearing animals treated with NP-BBNcomplexes [20mg/kg NP and 1.34µmoles BBNper animal(20mg/kg)]; Group 6: NP-SAN; tumor- bearing animals treated with NP-SANcomplexes [20mg/kg NP and 2.18µmolesSAN(20mg/kg)]and Group 7: NP-BBN-SAN; tumor-bearing animals treated with NP-BBN-SAN complexes [20mg/kg NP, 1.34µmoles BBN(20mg/kg), and2.18µmolesSAN(20mg/kg)]. After the treatment, the animals were sacrificed and collected blood as well as tissues for various analyses.

7.2.6 *Tumor regression and animal survival*

The tumor diameter was measured by vernier calipers and tumor volume was calculated [Jayakumar et al., 2009]. For the survival study, eight animals from selected groups were monitored for approximately one month and tumor volume was also calculated.

7.2.7 *Alkaline single cell gel electrophoresis (Comet assay)*

The cellular DNA damage following the treatments in tumor and liver tissues was analysed by comet assay [Cerda et al., 1997]. The comet parameters – tail length, tailmoment, %DNA in tail and olive tailmoment - were calculated using CASP software to convert the results into numerical values [Konca et al., 2003].

7.2.8 *Analysis of antioxidant status*

The levels of superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GPx) and lipid peroxidation in tumor and liver tissues of animals following various treatments were analysed. The SOD activity was measured according to McCard and Frederich method, 1969and the results were expressed as Units/mg Protein. The GSH in the samples react with dithiobis-2- nitrobenzoic acid (DTNB) and produces yellow coloured product on reduction with maximum absorption at 412nm [Moron et al., 1979]. The GPx assay was carried out based on the method described by Hafeman et al, 1974.The GPx in the sample degrades the H_2O_2 in presence of GSH. The remaining GSH was measured by its reaction with DTNB which produces a coloured product with maximum absorbance at 412nm. The lipid peroxides in the samples react with thiobarbituric acid in acetic acid solution and measured as malondialdehyde at 532nm [Janero, 1990; Buege and Aust, 1978]. The protein levels in the samples were analyzed by Lowry's method [Lowry et al., 1951].

7.2.9 *Serum biochemical parameters*

Blood samples were collected following the treatments by cardiac puncture and serum was seperated by centrifugation at 2500rpm for 15min. Serum creatinine level was measured by alkaline picric acid method [Bonsnes and Taussky, 1945]and serum glutamate oxaloacetate transaminase (SGOT) level was quantified according to the method of Thefeld*et.al.* in 1974.

7.2.10*Activity of tumor markers*

The activity of common solid tumor markers such as β-D glucuronidase (BDG), myeloperoxidase (MP) and lactate dehydrogenase (LDH) were studied. These are markers for tumor metastasis. The BDG and MP activity in tumor, liver and kidney tissues were analysed [Kawai and Anno, 1971; Desser et al., 1972]. The activity of LDH in tumor was also studied as tumors have higher lactate to pyruvate ratio [Wei Bhaar et al., 1975].

7.2.11*Gene expression study*

The transcriptional level expression of genes associated with tumor hypoxia and apoptotic cell death in tumor tissues were studied. The RNA was isolated by acid guanidium thiocyanate phenol- chloroform extraction method [Chomczynski and Sacchi, 2006]. cDNA was synthesized using 10µg of RNA and qRT- PCR was performed to amplify the

genes using specific primers. The data was presented as relative fold change in the transcription of genes based on the control [Schmittgen and Livak, 2008].

7.2.12 *Histopathology*

The tissues- tumor, liver and kidney- were embedded in paraffin wax after fixed in 10% formaldehyde solution. The sections with 0.5micron thickness were prepared and stained with hematoxylin- eosin (H&E) for the pathological evaluation [Culling, 1974].

7.2.13*Statistical analysis*

The results were presented as Mean ± SD and analyzed by GraphPad PRISM software version 5. Statistical analyses of the results were performed using ANOVA with Tukey-Kramer multiple comparisons test.

7.3 RESULTS AND DISCUSSION

7.3.1 *Effect of nanoparticle- drug complexes in tumor regression*

Figure 7.1presents the data on tumor regression following the administration of NP and NP-drug complexes to mice bearing solid tumor on hind limbs. The initial volume of the tumor in all animal groups was normalised to $1cm^3$ based on Control group for interpreting the results easily. In the control animals, the tumor volume increased from $1cm^3$ on day 1 to $1.57 \pm 0.08cm^3$ on day 12. The groups treated with NP, drugs and NP-drug complexes showed no increase in tumor volume, but recorded tumor regression to different extents. NP-BBN-SAN complexes showed the highest regression in tumor volume ($0.178 \pm 0.01cm^3$). This reduction in tumor volume ($p<0.001$) in NP-BBN-SAN group suggested the cytotoxic potential of these complexes to tumor. In the case of animals treated with BBN, NP-SAN as well as NP-BBN were showed reduction in tumor volume to $0.67 \pm 0.01cm^3$, $0.5 \pm 0.07cm^3$ and $0.4 \pm 0.05cm^3$ respectively, whereas the NPs and SAN treatment could not create much difference in tumor volume.

7.3.2 *Effect of NP and NP-drug complexes on cellular DNA of tumor tissues*

The tumor-bearing animals were administered with NP, drugs and NP-drug complexes. The tumors were excised on the 13^{th} day and the cellular DNA of the tumor tissues were analysed by alkaline comet assay to assess damage to DNA. The comet parameters- tail length, % tail DNA, tail moment and olive tail moment- were found to be increased ($p<0.05$) in tissues following treatment with NP-BBN-SAN complexes compared to the

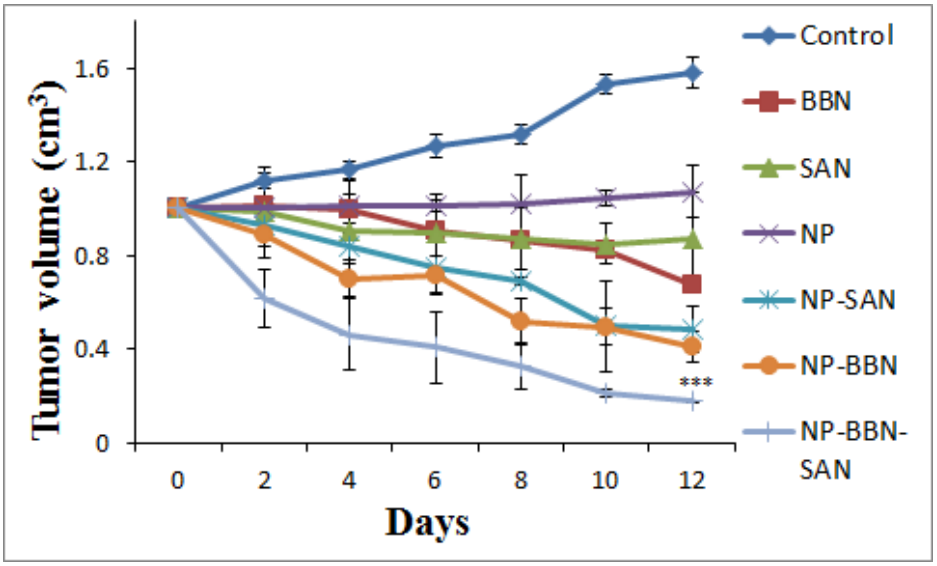

Figure 7.1: Tumor volume following various treatments. Note: The values are presented as mean± SD. The significance with p value <0.001 and <0.01 were observed in NP-BBN-SAN group compared to control and NP-BBN group respectively.

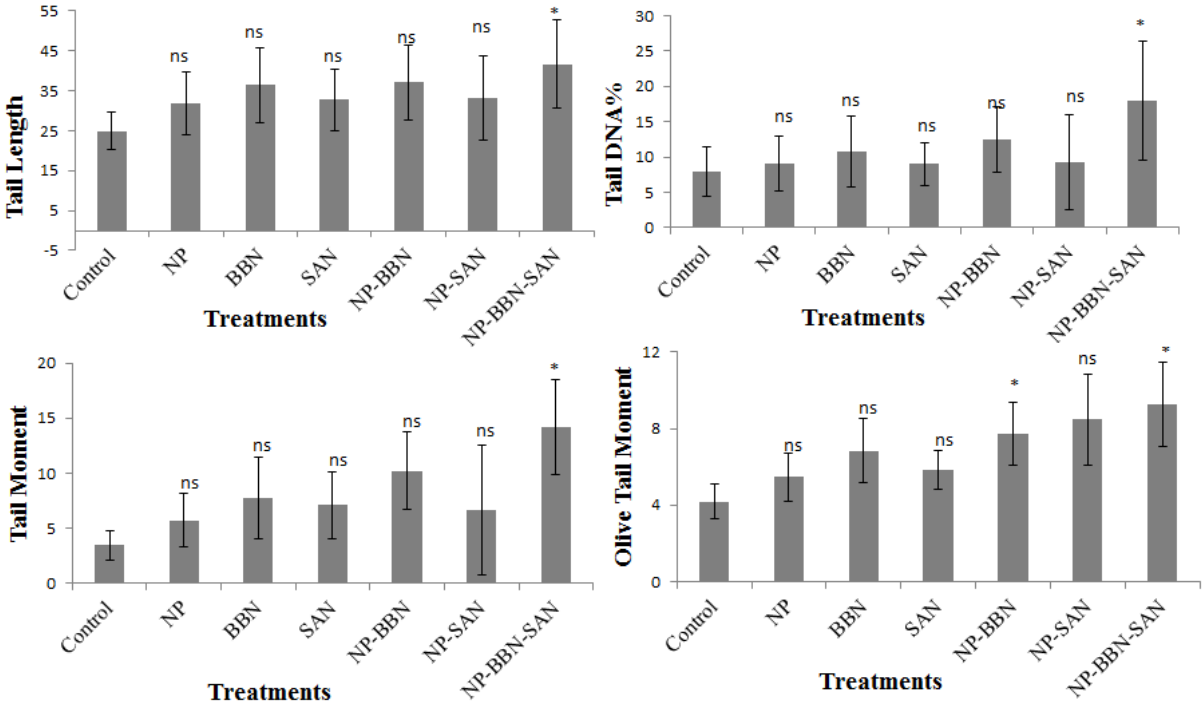

Figure 7.2: Comet parameters in tumor tissues after various treatments. Note: All values are presented as mean± standard deviation (SD). ^{ns} indicates non- significance (p>0.05) and * indicates significance (p<0.05) compared to the Control.

control and other groups of animals (figure 7.2), suggestive of induction of greater extend of damage to DNA by these complexes. There was no statistically significant variation in other treatments groups compared to the control.

7.3.3 *Effect of NP, drugs and NP-drug complexes in the expression of genes in tumor tissue*

The transcriptional level expression of genes- *hif-1α, vegf, akt, bax, bcl2, caspase 3, caspase 8,caspase 9*and *mmp2*- in control and following various treatments were analysed to reveal the mechanisms underlying regression of tumor. The expression of *hif-1α* and its target genes constitutea major adaptive hypoxia-response in solid tumor cells[Flamant et al., 2010]. The influence of NP- drug complexes in the transcription of *hif-1α* and its target genes were examined by qRT-PCR on the cDNA of the gene transcripts,using specific primers.

The transcription of the gene *hif-1α* was noticeably down regulated in NP-BBN-SAN treated groups compared to control with $p<0.001$. The down regulation in the expression of *hif-1α* can be seen in BBN, SAN, NP-BBN and NP-SAN group; however, these differences are not statistically significant in comparison with the control as evidenced from figure 7.3a. It has been reported that the activated *hif-1α* influences angiogenesis to support tumor growth through the expression of angiogenic factor VEGF [Masoud et al., 2015]. The expression of *vegf* was also investigated in all the treatment groups. It was found that the transcription of the gene *vegf* was down regulated significantly in SAN ($p<0.01$), NP-BBN ($p<0.01$), NP-SAN ($p<0.01$) and NP-BBN-SAN ($p<0.001$) groups compared to the control (figure 7.3b).

The PI3K/Akt signalling pathway plays a significant role in cancer cell growth and metastasis, and considered as a central target since it congregates several signalling pathways involved in tumor growth [Li et al., 2014; Meng et al., 2006]. Following the treatment with NP-BBN-SAN complexes and SAN showed significant down regulation in the transcription of *akt* compared to the control (figure 7.3c). As *akt* is involved in cell proliferation and metastasis, its down regulation by NP-BBN-SAN complexes suggests the therapeutic efficacy of the complexes.

Hypoxia has been shown to influence chemotherapy-induced apoptosis in cancer cells by regulating apoptotic pathways at the transcriptional and translational level [Sermeus et al.,

2012]. In the tumor tissues of animals treated with NP-BBN-SAN complexes, there was significant up regulation in the transcription of apoptotic gene *bax* and *caspase 8*, while anti-apoptotic gene *bcl2* was down regulated (figure 7.3d to 7.3g); suggesting the involvement of apoptosis in tumor regression. However, there was no significant increase in the transcription of *caspase 3* in NP-BBN-SAN treatment as evident from figure 7.3h.

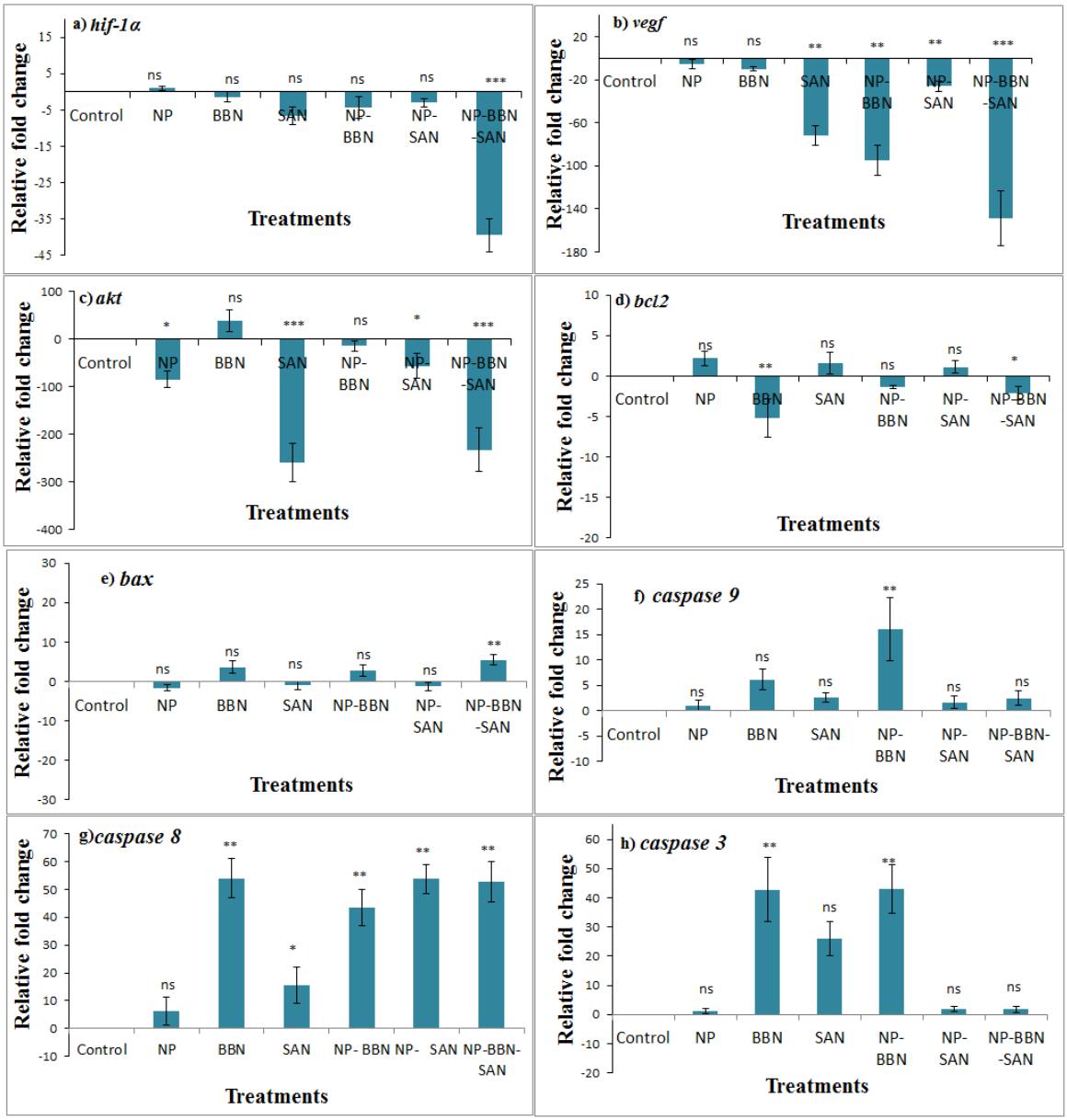

Figure 7.3: Effect of NP- drug complexes in the transcription of genes. Note: All values are presented as mean± standard deviation (SD). ^{ns} indicates non- significance ($p>0.05$) and *, ** and *** indicates significance with p value $p<0.05$, $P<0.01$ and $p<0.001$ respectively, compared to the control.

The transcriptional expression of *tnf-α* was also evaluated since it has an important role in apoptosis [Tran et al., 2009]. The transcription of *tnf-α* was significantly up regulated in NP-BBN-SAN group compared to the control as evidenced from figure 7.4b. There was a significant up regulation in the transcription of *tnf-α* in the animals administered with NP-BBN; however, more significant increase can be seen in the case of NP-BBN-SAN treated animals.

Matrix metalloproteinases (mmp) plays a significant role in cancer metastasis. The gene *mmp2* found to be involved in the degradation of extracellular matrix and enhances tumor migration [Mendes et al., 2007]. A significant down regulation in the expression of *mmp2* was observed in tumor tissues of animals treated with NP-BBN-SAN compared to control (7.4a), suggested the anti-metastatic potential of these complexes.

The apoptosis mechanism can be brought about through *caspase 8*/*caspase 3* pathway or *caspase 9*/*capase 3* pathway, both independent of *tnf-α*. Apoptosis can also be brought about through *tnf-α*/*caspase 8*/*caspase3* pathway as well as *tnf-α*/*caspase 8* pathway which is independent of *caspase 3*. The present result would suggests that in case of NP-BBN-SAN treated group the last pathway involving *tnf-α*through *caspase 8*, independent of *caspase 3*, could be operative. However, it can be seen in the figure 7.3 that there is significant up regulation of the genes for *caspase 9*, *caspase 8* and *caspase 3* in the groups treated with NP-BBN. This would suggest that in this particular group the two pathways of apoptosis - *caspase 9/3* (intrinsic) as well as *caspase 8/3* (extrinsic) - do operative simultaneously.

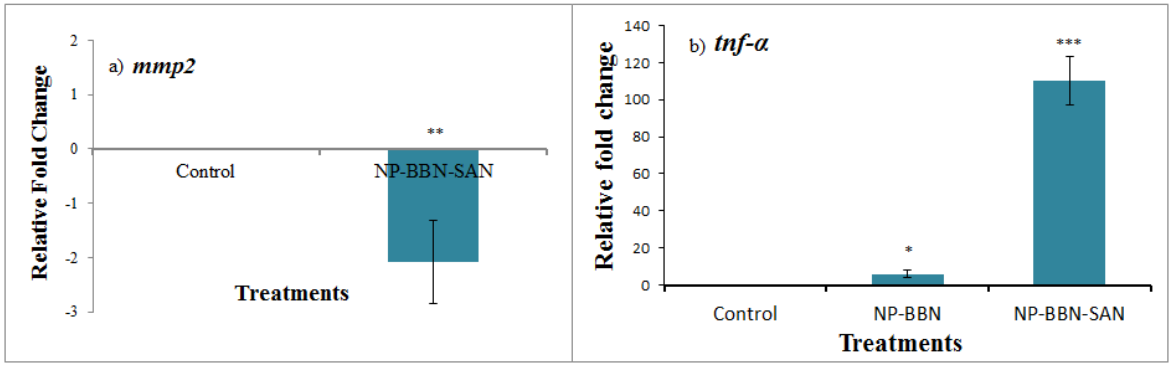

Figure 7.4: The relative fold change in the transcription of *mmp2* (a) and *tnf-α* (b). Note: The results were presented as Mean ± SD. *, ** and *** indicates significance with p value $p<0.05$, $P<0.01$ and $p<0.001$ respectively, compared to the control.

7.3.4 *Influence of BBN, SAN and NP-BBN-SAN complexes on antioxidant status of tumor and liver tissues*

The increased level of the major antioxidant, glutathione (GSH), in tumor is associated with cancer cell proliferation and metastasis [Carretero et al., 1999]. GSH can be considered as a main contributor of therapeutic drug resistance since it can interact with drugs and/or reactive oxygen species (ROS) generated by the cytotoxic drugs [Benlloch et al., 2005]. The GSH level was increased, >200nanomoles/mg protein, in tumor tissues of control animals. However, in tumor tissues of animals treated NP-BBN-SAN complexes showed a significant decrease, <100nanomoles/mg protein, in the GSH level compared to the control (figure 7.5b). NP-BBN treatment was also showed a decrease in GSH level while more inhibition could be seen in NP-BBN-SAN complex treatment.

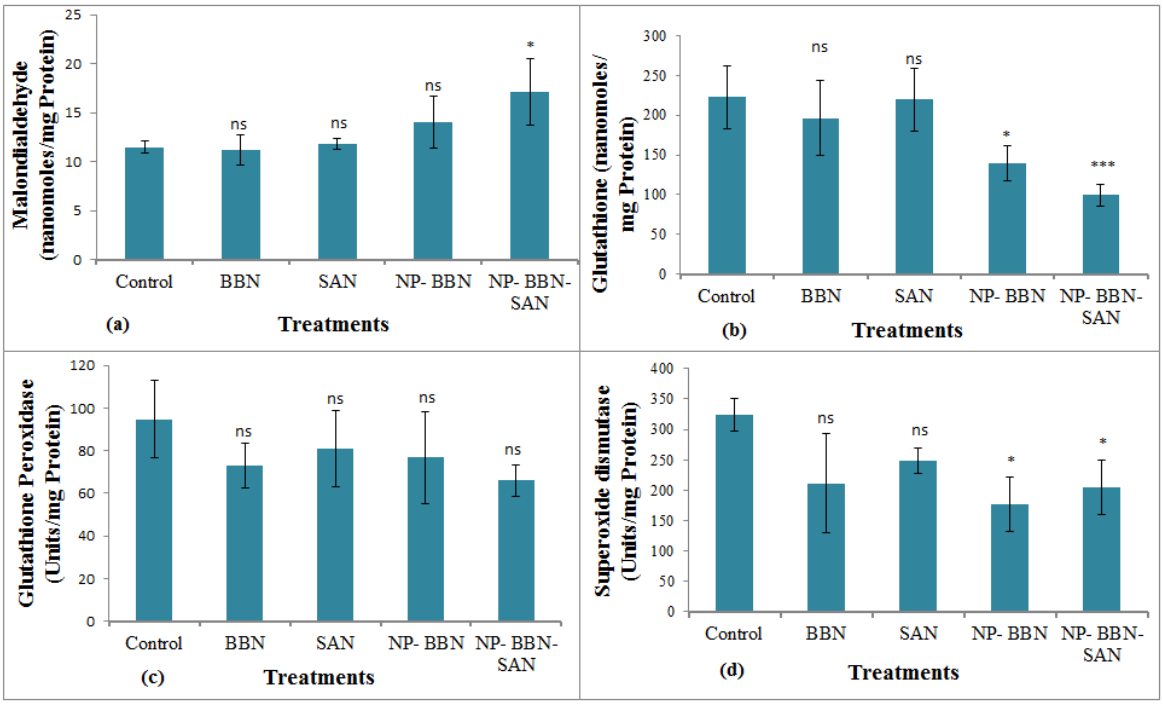

Figure 7.5: Effect of drugs and NP- drug complexes on antioxidant status in tumor tissues. a) MDA formation in tumor, b) Glutathione activity, c) Glutathione peroxidase activity and d) Superoxide dismutase activity. Note: All values are presented as mean± standard deviation (SD). ^{ns} indicates non- significance ($p>0.05$) and *and *** indicates significance with p value $p<0.05$ and $p<0.001$ respectively, compared to the Control.

In human breast cancer MCF-7 cells, the increased expression of antioxidant enzyme superoxide dismutase (SOD) was effective in scavenging the over produced superoxide radicals by the generation of hydrogen peroxide which activate matrix metalloproteinase

(MMP2) resulting in tumor metastasis [Zhang et al., 2002]. The present study revealed that the activity of SOD was decreased ($p<0.05$) in tumor tissues of animals treated with NP-BBN and NP-BBN-SAN complexes compared to the control (substantiated the finding presented in figure 7.4a) as evident from figure 9d. This would suggest that these complexes could effectively prevent tumor progression. There was no statistically significant variation observed in the activity of glutathione peroxidase (GPx) following the treatments compared to control (figure 7.5c).

The level of lipid peroxidation in terms of malondialdehyde (MDA) was studied [Schwartsburd and Lankin, 1994] to access the oxidative status in tumor tissues after these treatments. It was found that the MDA level was increased significantly in tumor tissues of animals treated with NP-BBN-SAN compared to the control (figure 7.5a), ascertaining the treatment resulted depletion of antioxidant status in tumors.

The antioxidant status in liver tissues of these treated animals was investigated to examine how specifically the treatments affect the tumor. The levels of antioxidant enzymes- GPx and SOD - as well as MDA were found to be unchanged even after the treatment with the complexes compared to control as depicted in figure 7.6a, 7.6c and 7.6d.

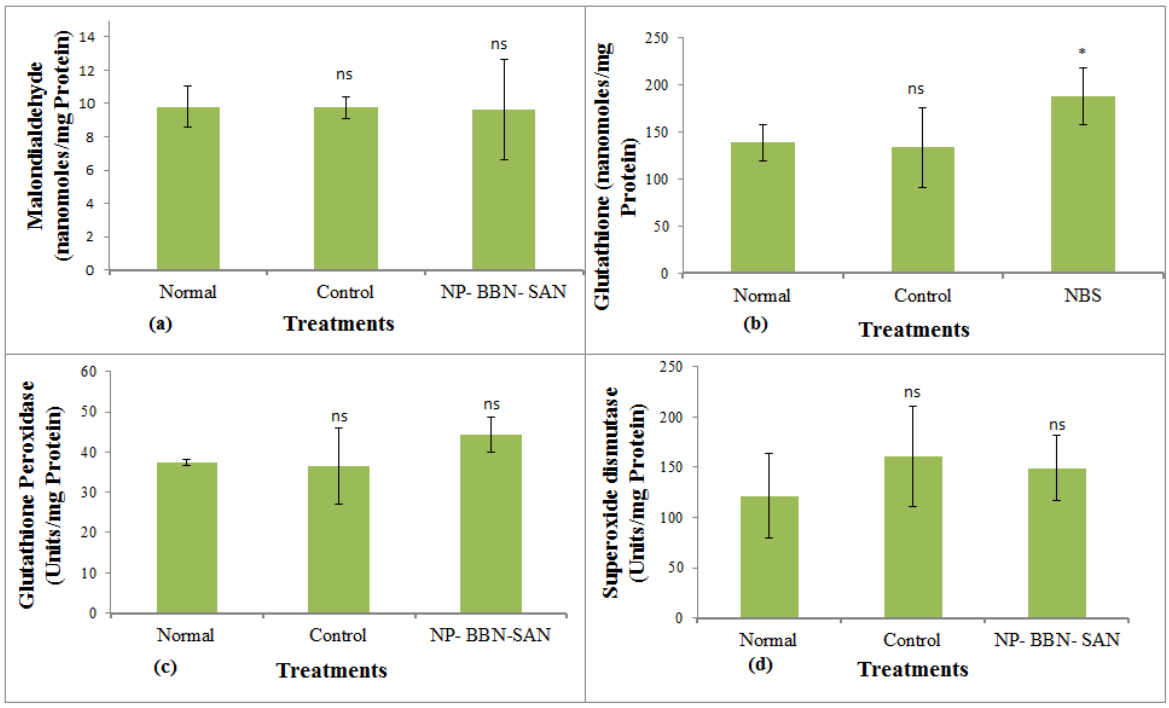

Figure 7.6: Effect of NP-drug complexes on antioxidant status of liver tissues. a) MDA formation in tumor, b) Glutathione activity, c) Glutathione peroxidase activity and d)

Superoxide dismutase activity. Note: All values are presented as mean± standard deviation (SD). ^{ns} indicates non- significance ($p>0.05$) and *indicates significance with p value $p<0.05$ compared to the control.

However, from the figure 7.6b it can be evidenced that a significant ($p<0.05$) increase in the GSH level in liver tissue after the treatment with NP-BBN-SAN complexes. This might be due to the effect of the treatment on GSH based detoxification in the liver, an important function of GSH.

7.3.5 *The activity of tumor markers*

The activity of tumor markers such as BDG and MP in tumor, liver and kidney tissues of Control, NP-SAN, NP-BBN and NP-BBN-SAN groups were examined. These enzymes possess important role in tumor progression and metastasis [Sperker et al., 2000; Mika and Guruvayoorappan, 2011]. The activity of BDG in tumor control was increased to approximately 10Units/mg protein while there were significant decrease (<8Units/mg protein) observed in tumor tissues after all treatments, compared to the control (figure $7.7A_1$). In both kidney and liver tissues, BDG activity was increased in control group; however, the treatment with the NP-drug complexes especially NP-BBN-SAN complexes reduced its activity nearer to the levels of normal animals without tumor (figure $7.7A_2$ and $7.7A_3$).

The level of MP in tumor, liver and kidney tissues were also evaluated. Unlike BDG, the MP level was significantly decreased only in NP-BBN-SAN treatment group compared to the control as evident from figure $7.7B_1$. The treatment caused no significant change in kidney and liver tissues compared to normal animals (figure $7.7B_2$ and $7.7B_3$). These results are further suggestive of specific antitumor activity of NP-BBN-SAN complexes.

7.3.6 **Assessment of Lactate dehydrogenase activity in tumor tissues**

The activity of LDH in tumor cells can be considered as a tumor marker since cancer cells follow anaerobic glycolytic pathway for their energy requirements [Pressley et al., 1992]. As presented in figure 7.8, the LDH activity was significantly reduced in all treatments compared to the control, indicating inhibition of anaerobic glycolysis. This can be correlated with the down regulation of *hif-1α*.

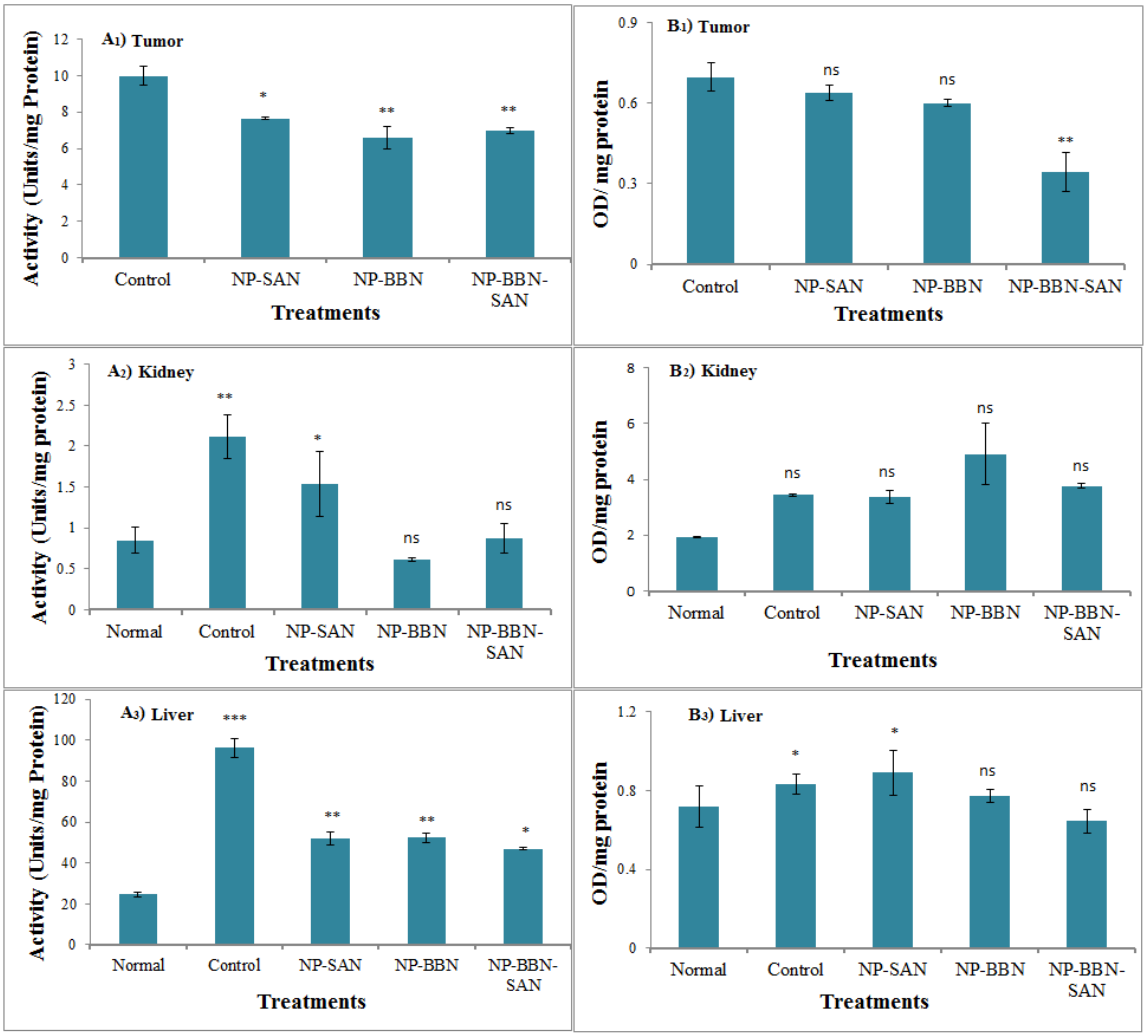

Figure 7.7: Effect of NP- drugs complexes on (A) β-D-glucuronidase and (B) myeloperoxidase. [#]

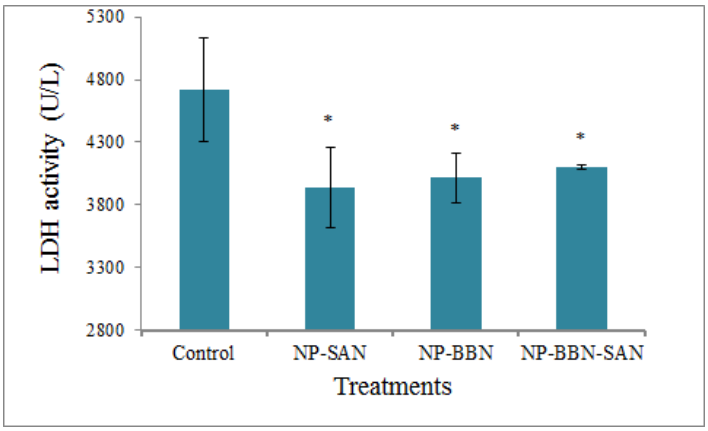

Figure 7.8: LDH activity in tumor following various treatments. [#]

[#]All values are presented as mean±SD. [ns] indicates non- significance ($p>0.05$) and [*and ***] indicates significance with $p<0.05$ and $p<0.001$ respectively, compared to the Control.

7.3.7 *Serum parameters*

The tumor-bearing animals recorded higher levels of serum creatinine and SGOT, which are markers of liver and kidney functions, compared to the normal animals without tumor. This could be due to the stress suffered by the animals due to the growing tumor. In tumor-bearing animals, the treatment with NP-BBN-SAN complexes reduced the levels of creatinine and SGOT (table 7.1), suggesting the therapeutic benefit of the complex.

Table 7.1: Serum creatinine and SGOT in control and following various treatments

Treatments	**Creatinine(mg/dl)**	**SGOT (U/L)**
Normal	0.455±0.05	142.93±7.1
Control	$0.865\pm0.06^{*}$	$213.66\pm7.5^{*}$
NP-BBN	$0.818\pm0.13^{*}$	144.63 ± 13.0^{ns}
NP-SAN	0.636 ± 0.02^{ns}	152.48 ± 23.7^{ns}
NP-BBN-SAN	0.637 ± 0.07^{ns}	171.67 ± 1.2^{ns}

Note: All values are presented as mean ± SD. ^{ns} indicates non- significance ($p>0.05$) and *indicates significance with p value $p<0.05$ compared to the control.

7.3.8 *Morphological changes in tissues*

The tissues - tumor, liver and kidney- were taken for the morphological examinations from control, NP-BBN and NP-BBN-SAN treated groups of animals.

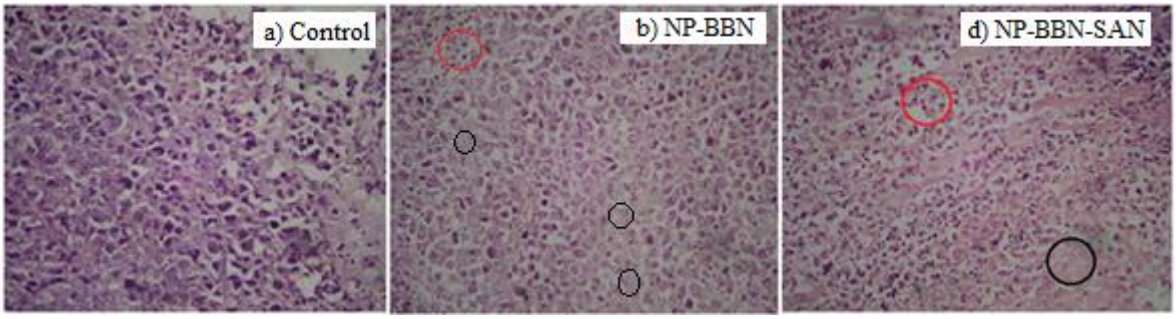

Figure 7.9: Tumor tissue morphology following the various treatments. The cells with condensed nuclei were marked with red circle and anucleated cells were marked with black circle. a) Control, b) NP-BBN and c) NP-BBN-SAN

As presented in figure 7.9, tumor control group showed a characteristic pleomorphism (figure 7.9a), while in NP-BBN-SAN group (figure 7.9c); areas with condensed nuclei - a

distinguishing feature of apoptosis, and region with anucleated cells - indicating necrosis - were discernible. It was also possible to visualize some changes in the cell morphology of NP-BBN treatment group; however the changes were more apparent in the group of animals treated with NP-BBN-SAN.

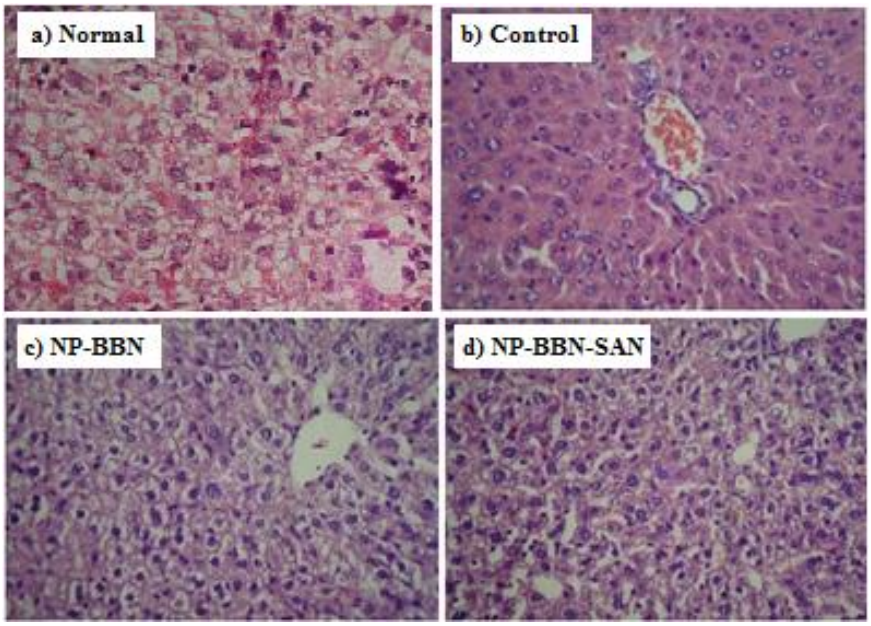

Figure 7.10: Morphology of liver tissues a) Normal (without tumor), b) Control (with tumor) l, c) NP-BBN and d) NP-BBN-SAN

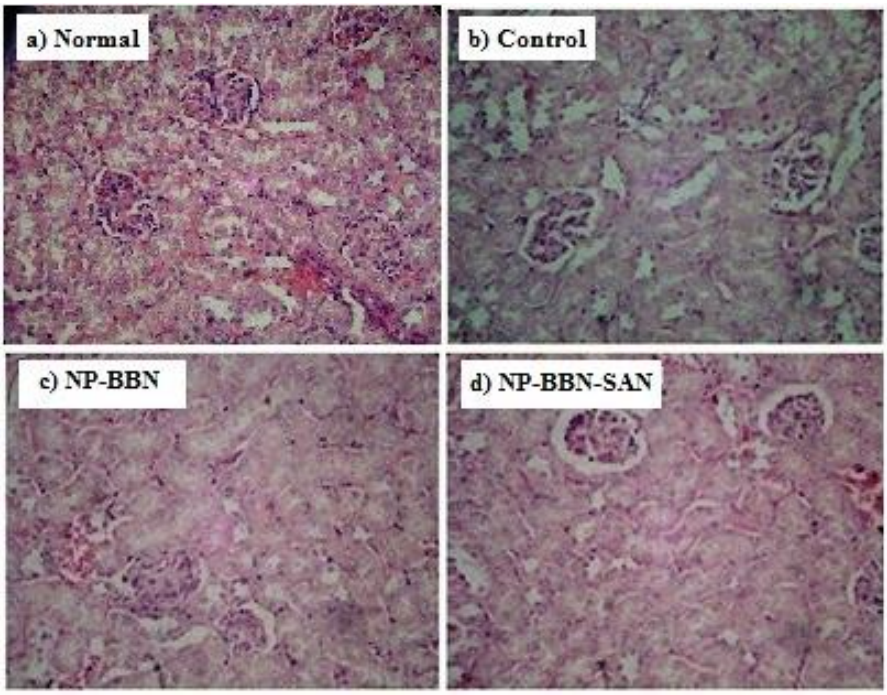

Figure 7.11: Morphology of kidney tissues a) Normal, b) Control, c) NP-BBN and d) NP-BBN-SAN

The increased presence of granulated cells can be seen in liver of control tumor-bearing animals (figure 7.10b). Following the treatment with NP-BBN-SAN complexes, these morphological changes were absent (figure 7.10d) and the morphology of the tissues was almost like that of the normal animal without tumor (figure 7.10a).In kidney tissues we could not find any significant changes in tissue morphology in control and following the treatments as it is evident from figure 7.11.

7.3.9 *Survival of tumor bearing animals and tumor regression*

The animals from control, NP-BBN and NP-BBN-SAN groups were kept under observation for approximately one month and percentage survival and tumor volume were monitored.

From the animal survival data on day 30 (figure 7.12), 100% survival can be seen in NP-BBN-SAN treated animals, while only 50% survival in NP-BBN and <20% in control animals. From these animals, tumor volume data was prepared and presented in figure 7.13. It was found that NP-BBN-SAN treatment could completely eradicated the tumor; however, in control and NP-BBN animals it is increased to 26 ± 0.4cm^3 and 6.8 ± 0.08cm^3 respectively on 30th day. These results authenticated the effect of NP-BBN-SAN complexes in the complete removal of the tumor without causing tumor recurrence for 30days, implying the therapeutic efficacy of the NP-BBN-SAN complexes.

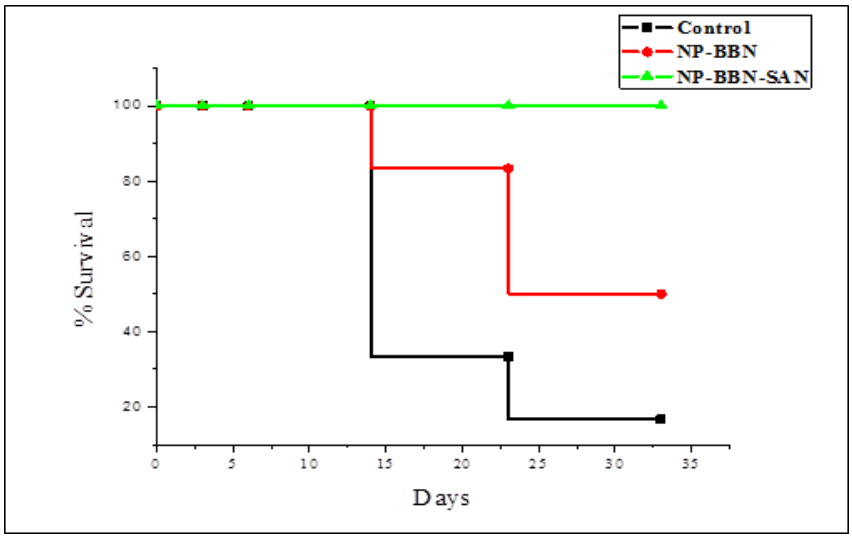

Figure 7.12: Percentage survival of the animals following the treatments with NP-BBN and NP-BBN-SAN.

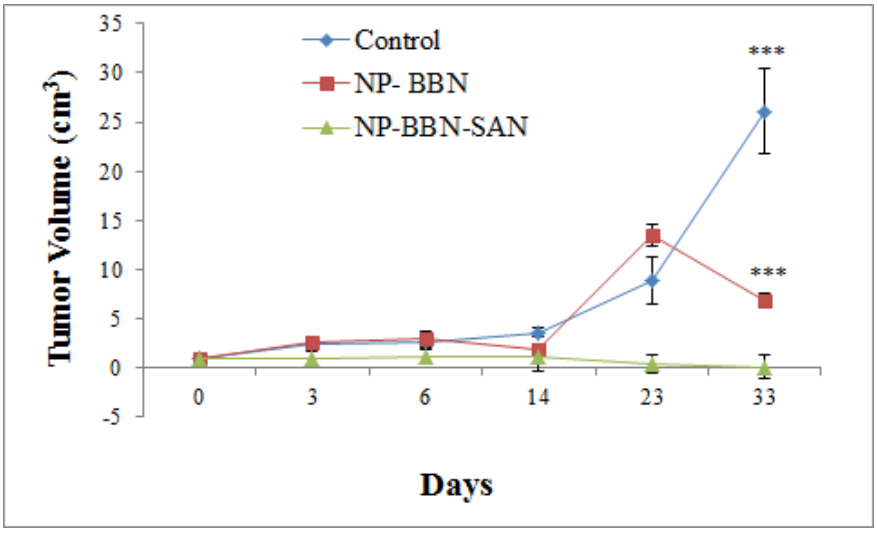

Figure 7.13: Tumor volume of animals kept under observation for one month following the treatment with NP-BBN and NP-BBN-SAN. Note: All values are presented as mean±SD. *** indicates significance with p value $p<0.001$ compared to NP-BBN-SAN treated group.

7.4 CONCLUSION

In the present study, the NP- BBN complexes were specifically delivered to tumor with the help of SAN by exploiting the tumor hypoxia. The NP-BBN-SAN complexes were found to be more effective in reducing tumor volume compared to the other treatment groups and control. The mechanism behind the tumor regression can be ascribed to i) the down regulation in the transcription of *hif-1α* and its associated genes- *vegf* and *akt*- involved in angiogenesis and cell proliferation, thereby results in tumor regression and ii) *tnf-α* induced extrinsic pathway of apoptosis through *caspase 8*, although other apoptotic pathways may be involved which requires further investigation. The tumor targeted specific effect of NP-BBN-SAN complexes was evident from the observations on tissue morphology, serum parameters, tissue antioxidant levels and tumor markers. The studies suggest the potential applicationand therapeutic relevance of NP-BBN-SAN complexes in tumor control. This chemo-directed specific targeting to hypoxic tumor is a potential therapeutic strategy of clinical relevance. However, more studies with different tumor models may be needed before undertaking clinical trials.