CHAPTER-5

EXPERIMENT-I

5.0. EXPERIMENT-I

Efficacy assessment of NAC for the correction of sodium arsenite instigated hepatic and reproductive oxidative stress via *in vitro* approach.

5.1. Objectives of the experiment

This study was considered for evaluating the direct accomplishment of NAC on sodium arsenite driven hepatic and utero-ovarian ailments e.g. lipid peroxidation, antioxidant status and DNA damage sustaining in the *in vitro* state.

5.2. Study framework

The experimental models were anesthetized using HCl ketamine (24 mg/kg body weight) as per the procedure proposed by animal ethical board of the concern institution (IEC/7-6/C-6/16 dated 26.8.16) and euthanized using barbiturate overdose (≥86 mg/kg body weight). Then the samples of liver, uterus and ovary tissues were removed and placed into the freezer at -20˚C using germ-free separate bags. Then obtained samples were weighted and rinsed with chilled Krebs solution and finally distributed in several groups and 3 hrs and 6 hrs exposure of incubation time were followed.

Figure 5.1

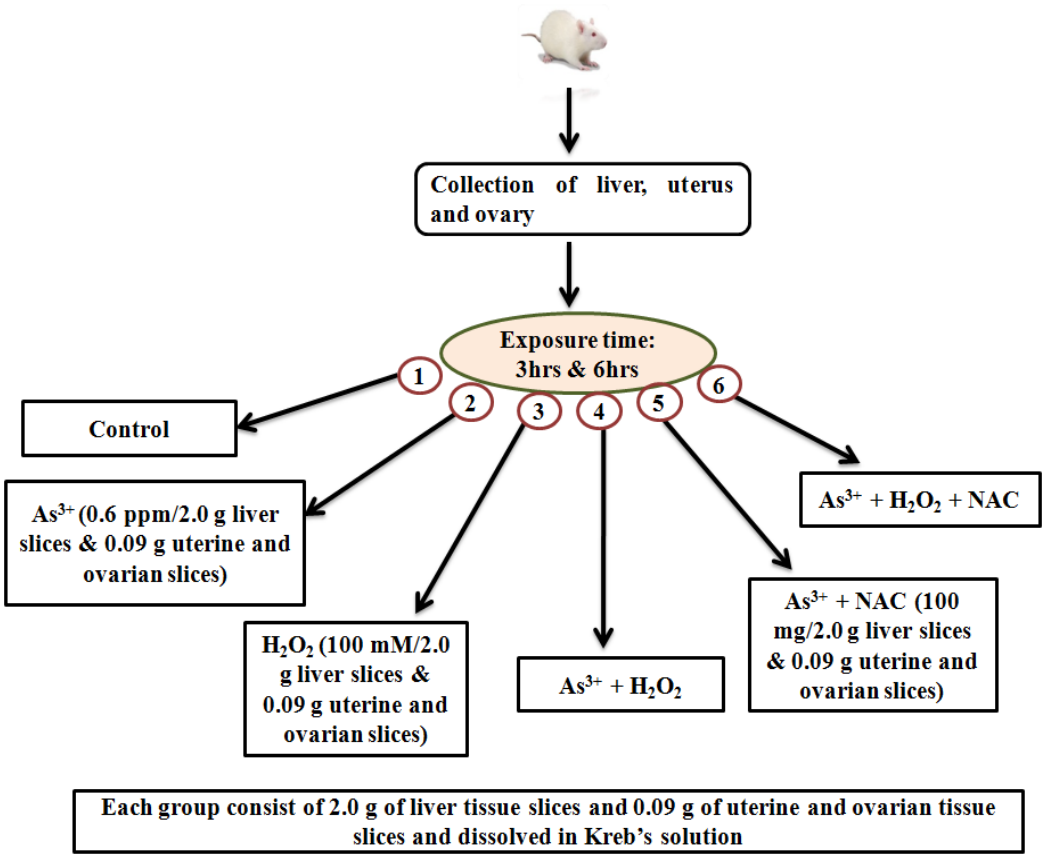

Fig. 5.1: Study design and group distribution of *in vitro* experiment.

Here six (6) groups were considered for the experimentation. Each group consisted of 2.0 g of liver slices and 0.09 g of ovary and uterus tissue slices which were immerged in 20 ml of Kreb's solution. Group 1 was vehicled control group wherein 2.0 g of liver slices and 0.09 g of ovary and uterus tissue slices were incubated; group 2 was sodium arsenite incubated group in which 0.6 ppm sodium arsenite was treated per 2.0 g of liver slices and 0.09 g of ovary and uterus tissue slices; group 3 was H_2O_2 treated group wherein 100 mM of H_2O_2 was incubated as per same tissue concentration of group 1; group 4 was sodium arsenite and H_2O_2 incubated group and here the tissue concentration was also same as group 1; group 5 was sodium arsenite and NAC incubated group where NAC was given at 100 mg per 2.0 g of

liver slices and 0.09 g of ovary and uterus tissue slices; group 6 was sodium arsenite, H_2O_2 and NAC treated group.

5.3. Composition of Krebs solution

Sodium chloride (NaCl) = 8.0 g

Potassium chloride (KCl) = 0.20 g

Calcium chloride ($CaCl_2$) = 0.20 g

Magnesium chloride ($MgCl_2$, $6H_2O$) = 0.10 g

Sodium bicarbonate ($NaHCO_3$) = 1.0 g

Disodium biphosphate (Na_2HPO_4) = 0.05 g

Glucose = 1.0 g

Distilled water = 1.0 litre

5.4. Results

5.4.1. Effects on lipid peroxidation via duration dependent manner

There was noteworthy development of MDA and CD level due to peroxidation of lipid following arsenic treatment in the hepatic and reproductive tissues (Table 5.1) in both 3 hrs and 6 hrs exposure. In the similar direction exposure of only H_2O_2 in both exposures time also augmented the level of these lipid peroxidation products significantly (Table 5.1). Indeed, similar style of results was observed when hepatic and ovarian-uterine tissues were exposed to As^{3+} and H_2O_2 in both incubation times. However, 6 hrs incubation time showed comparatively worst outcome in the levels

of these lipid peroxidation markers when make a judgment with control group ($p<0.001$). Treatment of NAC in arsenicated group significantly reversed back the MDA level in uterine-ovarian and hepatic cells especially in 6 hrs incubation time ($p<0.05$) whereas 3 hrs incubation exhibited mild recovery which was insignificant. Approximately 1.2 fold (both for uterine-ovarian tissues) and 1.11 fold (hepatic tissue) recovery was noticed when sodium arsenite treated animal models were incubated with NAC for 3 hrs wherein 1.27, 1.43 and 1.18 fold correctional changes were apparent in 6 hrs incubation respectively. In CD, NAC showed insignificant recovery in 3 hrs incubation time and exhibited 1.1, 1.24 and 1.14 fold down production accordingly in uterine, ovarian and hepatic tissues, only 6 hrs exposure of NAC in arsenicated group showed better and significant result (Table 5.1, $p<0.05$) by appearing 1.47, 1.48 and 1.33 fold recovery respectively.

Table 5.1

Uterine MDA & CD				
	MDA(nmole/gm)		**CD(nmole/gm)**	
Groups	**3 hrs**	**6 hrs**	**3 hrs**	**6 hrs**
Control	12.11±1.28	12.43±0.53	9.56±0.23	8.94±0.42
As^{3+} (0.6 ppm)	15.14±1.15*	17.43±1.2*	12.56±0.78*	14.65±1.17*
H_2O_2 (100 mM)	18.58±0.97*/#	18.57±0.61*/#	12.68±0.65*	18.65±1.12**/#
As^{3+}+ H_2O_2	20.45±1.677***/#	24.28±1.42***/##	13.69±1.10*	20.32±1.25***/##
As^{3+}+ NAC (100mg)	12.71±1.13	13.74±1.15#	11.43±0.48	9.94±0.49#
As^{3+}+ H_2O_2 + NAC	13.46±0.89	14.83±0.74#	11.76±0.57	10.43±0.52#
Ovarian MDA & CD				
Control	9.87±0.52	9.64±0.43	5.53±0.20	5.65±0.19
As^{3+} (0.6 ppm)	12.65±0.39*	14.47±1.12**	7.22±0.24*	9.93±0.54**
H_2O_2 (100 mM)	12.73±1.0*	14.83±1.26**	9.63±0.43**/#	10.48±0.36**
As^{3+}+ H_2O_2	17.61±0.68*/#	18.38±1.43***/#	9.83±0.38**/#	12.94±0.68***/#
As^{3+}+ NAC (100mg)	10.54±0.39	10.14±0.84#	5.86±0.21	6.73±0.27#
As^{3+}+ H_2O_2 + NAC	10.65±0.47	11.47±0.46	7.83±0.32	6.93±0.42
Hepatic MDA & CD				
Control	28.84±1.25	29.48±1.22	23.52±0.53	22.72±0.39
As^{3+} (0.6 ppm)	32.84±1.13*	35.76±0.59*	26.83±0.83*	34.18±1.24**
H_2O_2 (100 mM)	33.43±1.11*	38.83±1.17**	28.73±0.62*	33.74±1.18*
As^{3+}+ H_2O_2	35.84±1.13*	41.58±1.28***	31.83±1.21**/#	36.29±0.51***
As^{3+}+ NAC (100mg)	29.56±1.10#	30.23±0.82#	23.64±0.71	25.82±0.42#
As^{3+}+ H_2O_2 + NAC	29.64±0.86	32.84±0.94	23.86±0.88#	28.53±1.17#

Table 5.1: Consequence of NAC in antagonizing sodium arsenite derived MDA plus CD level on hepatic and reproductive tissues via duration dependent mode. The results represent Mean ± SE (Standard Error), n=6. Data were evaluated using one-way-ANOVA following Dunnett's test (post-hoc). *$p<0.05$, **$p<0.01$ and ***$p<0.001$ were considered for significance analysis when compared between

control and As^{3+}, H_2O_2, and As^{3+}+ H_2O_2 group, whereas #p<0.05 and ##p<0.01 were considered for comparison between As^{3+} and rest of the additional groups.

5.4.2. Antioxidant Status by spectrophotometry

The enzymatic function of SOD was significantly suppressed following 3 hrs exposure of H_2O_2 and As^{3+}+H_2O_2 group in uterine tissue (Fig. 5.2; A) whereas, As^{3+} plus As^{3+} in association with H_2O_2 treatment in ovarian tissue significantly diminished SOD activity (Fig. 5.2; D) compared to control group. However, liver tissue did not illustrate any significant diminution of SOD activity due to 3 hrs exposure of sodium arsenite (Fig. 5.2; G). Moreover, 6 hrs exposures of As^{3+}, H_2O_2 and As^{3+} + H_2O_2 caused significant down regulation of SOD activity in all tissues (Fig. 5.2; A, D, G). Exposure of NAC in As^{3+} and H_2O_2 treated group significantly enhanced the SOD activity in utero-ovarian and hepatic tissue only on 6 hrs incubation (Fig. 5.2; A, D, G). About 1.1 and 1.5 fold up-gradation of SOD activity was observed following 3 hrs incubation, while about 1.3 and 1.73 fold increase was perceived in 6 hrs exposure of As^{3+}+NAC group comparing with As^{3+} and As^{3+} + H_2O_2 group respectively in uterine tissues. In ovarian tissues, incubation of NAC in arsenicated group for 3 hrs revealed insignificant but mild increase of SOD activity and about 1.07 and 1.09 fold recovery was achieved in As^{3+} and As^{3+} + H_2O_2 group wherein exposure for 6 hrs noticed 1.26 and 1.6 fold significant recovery in these respective groups. Similar category of outcome was also revealed in hepatic tissues for 3 hrs incubation of NAC in As^{3+} and As^{3+} + H_2O_2 group which was mild but insignificant. But 6 hrs incubation periods with NAC in arsenicated group focused significant results with 1.46 and 1.56 fold enhancement of SOD activity in above mentioned groups respectively.

A similar fashion of activity was perceived for catalase where 6 hrs incubation suppressed catalase activity drastically in all the groups of all the tissues (hepatic, ovarian and uterine) due to sodium arsenite (Fig. 5.2; B, E, H). Considering the uterine tissues, near about 1.2 and 1.1 fold up-gradation was recorded in As^{3+} + NAC and As^{3+} + H_2O_2 + NAC group respectively when compared with As^{3+} group continued 3 hrs incubation (Fig. 5.2 B). During 6 hrs exposure As^{3+} + NAC and As^{3+} + H_2O_2 + NAC group illustrated respectively 1.52 and 1.46 fold significant increments in respect of As^{3+} group. In ovarian tissues 3 hrs exposures exhibited 1.08 and 1.06 fold recovery and 6 hrs incubation elucidated about 1.4 and 1.34 fold improvement in As^{3+} + NAC and As^{3+} + H_2O_2 + NAC group correspondingly compared to arsenicted group (Fig. 5.2 E) which was significant. The incubation of sodium arsenite with hepatic tissues illuminated the same outcome like utero-ovarian tissues where 3 hrs incubation only have mild but insignificant reduction of catalase activity. But As^{3+} + H_2O_2 group showed noteworthy reduction with respect to control whereas 6 hrs incubation visualized more prominence (Fig. 5.2 H). Around 1.0 fold up-gradation of catalase activity was found in both As^{3+} + NAC and As^{3+} + H_2O_2 + NAC group in respect of arsenicated group following 3 hrs incubation, though 6 hrs incubation revealed 1.34 fold noteworthy correction in both the above mentioned group rather than As^{3+} incubated group (Fig. 5.2 H).

Considering GPx, treatment of As^{3+}, H_2O_2 and As^{3+} + H_2O_2 remarkably reduced the workability of this enzyme with respect of control following the incubation of 3 hrs only in reproductive tissues (Fig. 5.2; C, F, I). But following the 6 hrs exposure, all types of tissues in the experiment showed suppressed activity of GPx (Fig. 5.2; C, F, I). Incubation with NAC re-stimulated the GPx activity towards normalcy. In uterine

tissue, around 1.1 fold enhancement was seen in both the As^{3+} + NAC and As^{3+} + H_2O_2 + NAC group in contrary to arsenicated group during 3 hrs incubation while about 1.32 fold augmentation was apparent during 6 hrs incubation of above two groups (Fig. 5.2 C). Considering ovarian tissue around 1.3 and 1.1 fold recovery and 1.7 and 1.6 fold up-surge was monitored in 3 hrs and 6 hrs incubation respectively in As^{3+} + NAC and As^{3+} + H_2O_2 + NAC group comparing with arsenite treatment (Fig. 5.2 F). Incubation of hepatic tissues for 3 hrs augmented GPx status about 1.0 fold in both the groups (As^{3+} + NAC and As^{3+} + H_2O_2 + NAC) wherein about 1.5 and 1.45 fold revitalization was detected respectively after 6 hrs incubation period in these groups following arsenication (Fig. 5.2 I).

Therefore, above mentioned percentage of recovery elucidated that in all the instances introduction of NAC for 6 hrs was more efficient to bring back the capacity of SOD, catalase and GPx enzymes to their normal mode of action. In most of the conditions NAC application in only As^{3+} treated group showed more effective benefits as shown from the fold recovery rather than the combination group with As^{3+}+ H_2O_2 + NAC.

Figure 5.2

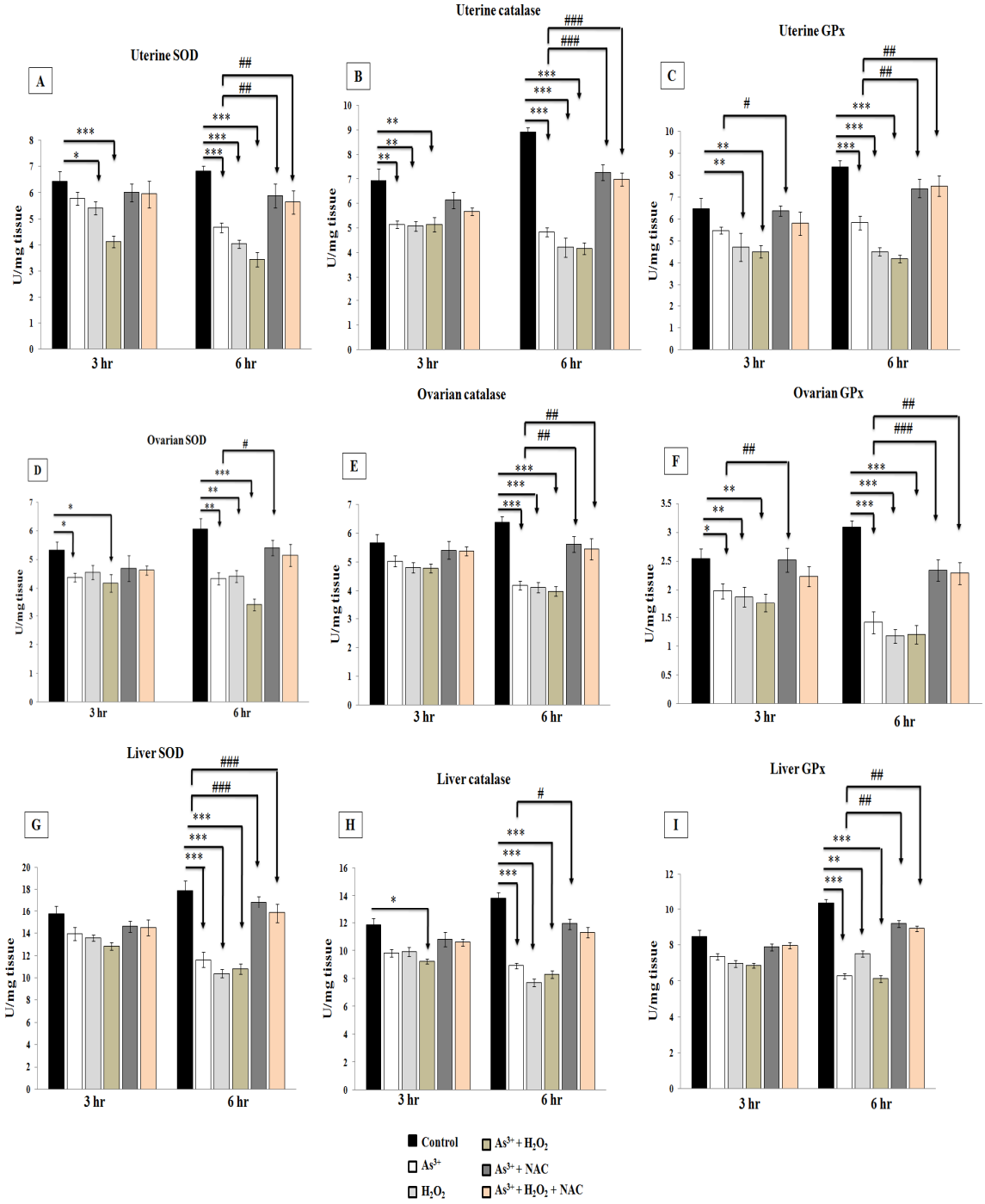

Fig. 5.2: The time dependent functioning of NAC in As^{3+} and H_2O_2 induced antioxidant enzyme activities in uterus, ovary and hepatic tissues. The results represent Mean ± SE (Standard Error), n=6. Data were evaluated using one-way-

ANOVA following Dunnett's test (post-hoc). *p<0.05, **p<0.01 and ***p<0.001 were considered for significance analysis when compared between control and As^{3+}, H_2O_2, and As^{3+}+ H_2O_2 group, whereas #p<0.05, ##p<0.01 and ###p<0.001 were considered for comparison between As^{3+} and rest of additional groups.

5.4.3. Evaluation of antioxidant status by native gel

The expressional activity of the antioxidant enzymes (SOD, catalase and GPx) was further evaluated on native electrophoretic gel. As^{3+}, H_2O_2 and As^{3+} + H_2O_2 showed remarkable unfavourable effects on these enzymes during 3 hrs and 6 hrs incubation in all the tissue (Fig. 5.3; A, B, C, D, E). However, 6 hrs incubation reflected more deleterious effect with reducing band mass with respect to 3 hrs incubation in majority of enzyme expression. Only for uterine SOD expression and hepatic SOD and catalase band densities were showing less intensify following 6 hrs incubation than that of 3 hrs in As^{3+} + NAC and As^{3+} + H_2O_2 + NAC groups (Fig. 5.3 A, B, C & D). From the zymogram, it has been illustrated that giving NAC on arsenicated and arsenic-H_2O_2 combined groups concomitantly up-surged the enzymatic expression which was evidenced from the higher band width measured as % density with respect to control (Fig. 5.3; A, B, C, D, E). Therefore, a time dependent consequence of toxicity was recognized in the appearance of the above said enzymes. Though only uterine catalase and GPx expression and hepatic GPx expression (As^{3+} + NAC) were showing duration dependent recovery by NAC (Fig. 5.3; A, B, C, D).

Figure 5.3

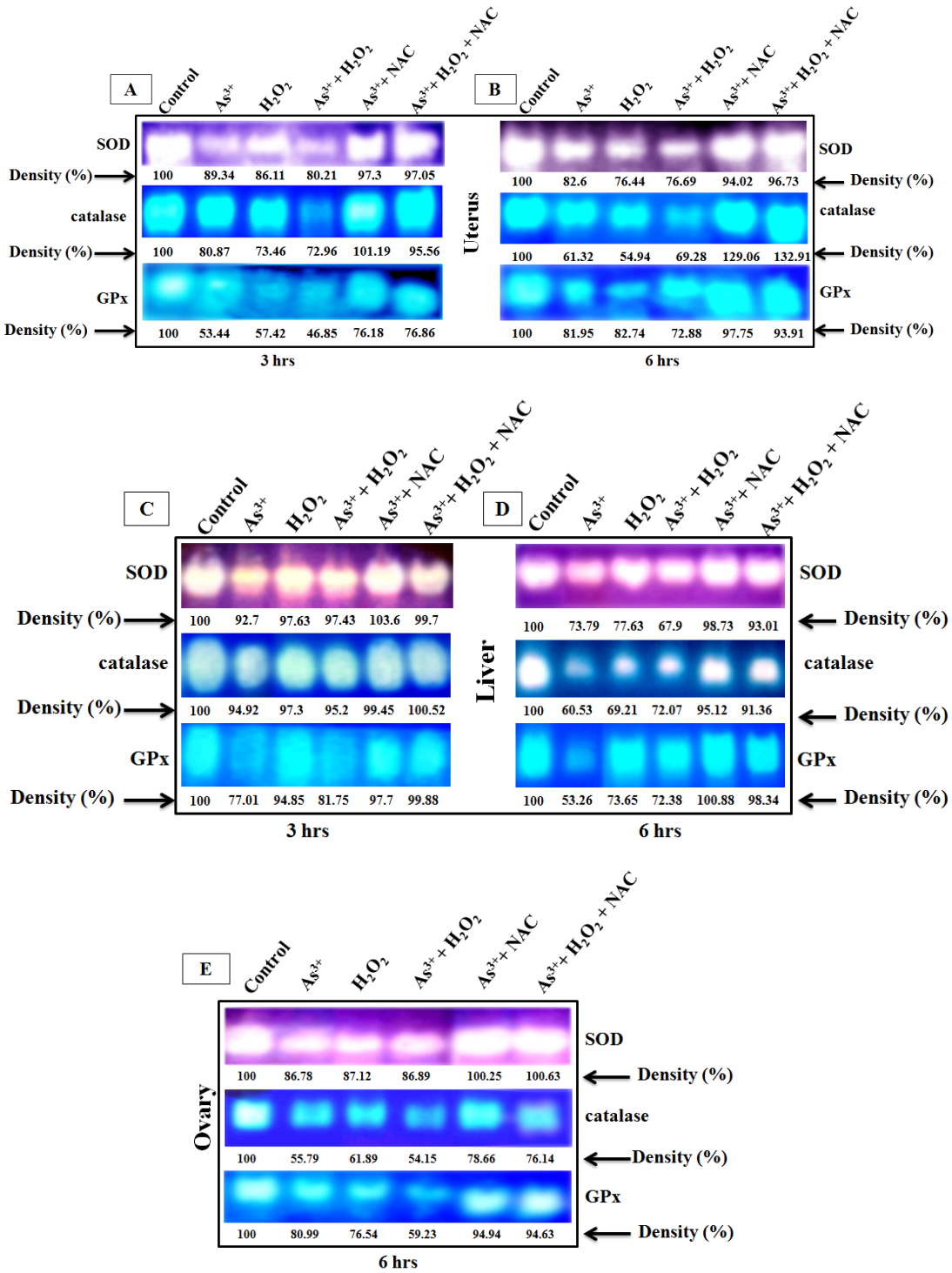

Fig. 5.3: The functioning of NAC in As^{3+} and H_2O_2 driven antioxidant enzyme expression on native gel in uterine-ovarian and hepatic tissues. The band width was evaluated as % density in respect of control.

5.4.4. Evaluation of steroidogenesis in ovary

The ovarian steroidogenesis was assayed through the measuring of Δ^5, 3β-HSD and 17β-HSD. A noteworthy devastating condition was seen in the functional status of these enzymes when ovarian tissue was kept with As^{3+}, H_2O_2 alone and As^{3+}+ H_2O_2 jointly (Fig. 5.4; A and B) for 3 hrs and 6 hrs. When NAC was given in As^{3+} group alone or combinely with $As^{3+}$and H_2O_2, the ovarian steroidogenesis was improved extensively to the normal. Considering the Δ^5, 3β-HSD activity, 3 hrs incubation explored 1.5, 1.38 and 1.55 fold noteworthy diminution in As^{3+}, H_2O_2, and As^{3+}+ H_2O_2 groups respectively with respect to control (Fig. 5.4 A). When NAC was incubated for 3 hrs in As^{3+} supplied group about 1.26 fold significant recovery was noted than only As^{3+} supplied group whereas 1.22 fold improvement was manifested in combined group of As^{3+}+ H_2O_2 + NAC with respect of As^{3+} + H_2O_2 group. Incubation for 6 hrs appeared 2.3, 1.82 and 2.14 fold inhibition following As^{3+}, H_2O_2, and As^{3+}+ H_2O_2 group accordingly rather than control (Fig. 5.4 A). NAC amplification for 6 hrs in only arsenicated group improved the functional status of 2.05 fold and in As^{3+} + H_2O_2 group revealed 1.68 fold improvements. Taking into account of 17 β-HSD, the functional status of this enzyme was dropped about 1.5, 1.4 and 1.52 fold following As^{3+}, H_2O_2, and As^{3+} + H_2O_2 group during 3 hrs exposure with respect to control (Fig. 5.4 B) while in 6 hrs such declining activity was about 2.06, 1.75 and 1.87 fold respectively in above said groups. When NAC was allowed to incubate in only arsenicated group for 3 hrs a 1.44 fold improvement was achieved and in As^{3+} + H_2O_2 group this recovery was 1.4 fold wherein 6 hrs incubation period denoted 1.98 fold and 1.73 fold betterment respectively in these group. Therefore, the fold recovery and the figure given below illustrated that NAC

treatment for 6 hrs was better when comparison made with 3 hrs exposure (Fig. 5.4; A and B).

Figure 5.4

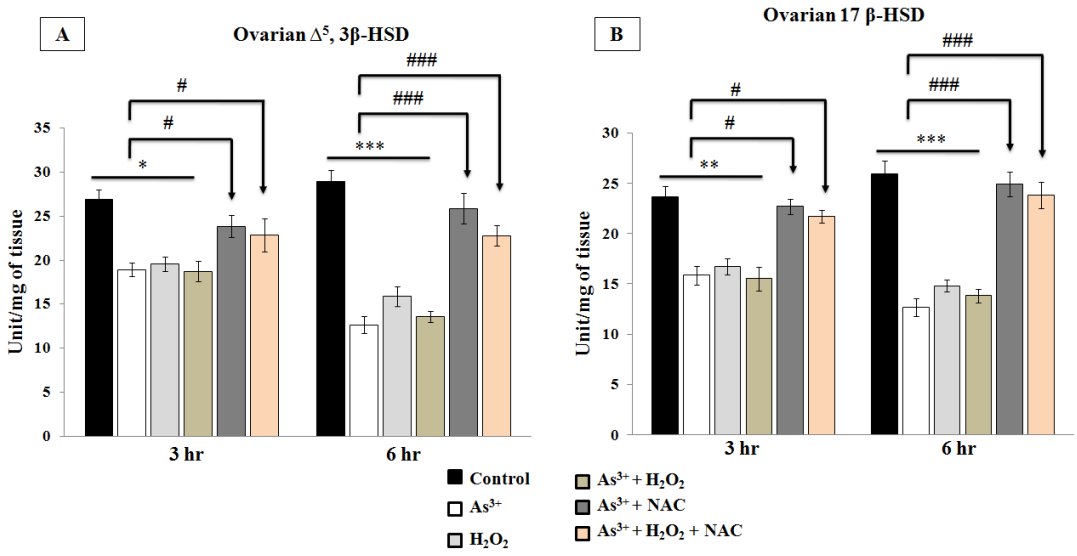

Fig. 5.4: The function of NAC with respect to As^{3+} and H_2O_2 driven steroidogenesis in ovary. The results represent Mean ± SE (Standard Error), n=6. Data were evaluated using one-way-ANOVA following Dunnett's test (post-hoc). *p<0.05, **p<0.01 and ***p<0.001 were considered for significance analysis when compared between control and As^{3+}, H_2O_2, and As^{3+}+ H_2O_2 group, whereas #p<0.05 and ###p<0.001 were considered for comparison between As^{3+} and rest of additional groups.

5.4.5. Comet assay

Arsenic and H_2O_2 both are accountable for hepatic DNA damage which was evidenced from comet assay from the variation of time. The rising number of comet cells along with increasing tail length were found in As^{3+} and H_2O_2 separately or conjointly during both incubation period (Table 5.2; Fig. 5.5; A and B). Moreover, such increasing nature of comet cells plus tail length was massive in 6 hrs exposure

(Table 5.2). This single cell lysis was postponed when NAC was applied in As^{3+} given group alone and arsenic conjointly with H_2O_2 group in both exposure period (Fig. 5.5; A and B; Table 5.2) though the functional efficacy of NAC was more promising when it was given alone with arsenic. Table 5.2 clearly confirmed that 6 hrs incubation period of NAC especially in arsenicated group was more promising and capable of lessening the amount of comet plus tail length in hepatic tissue than 3 hrs incubation.

Figure 5.5

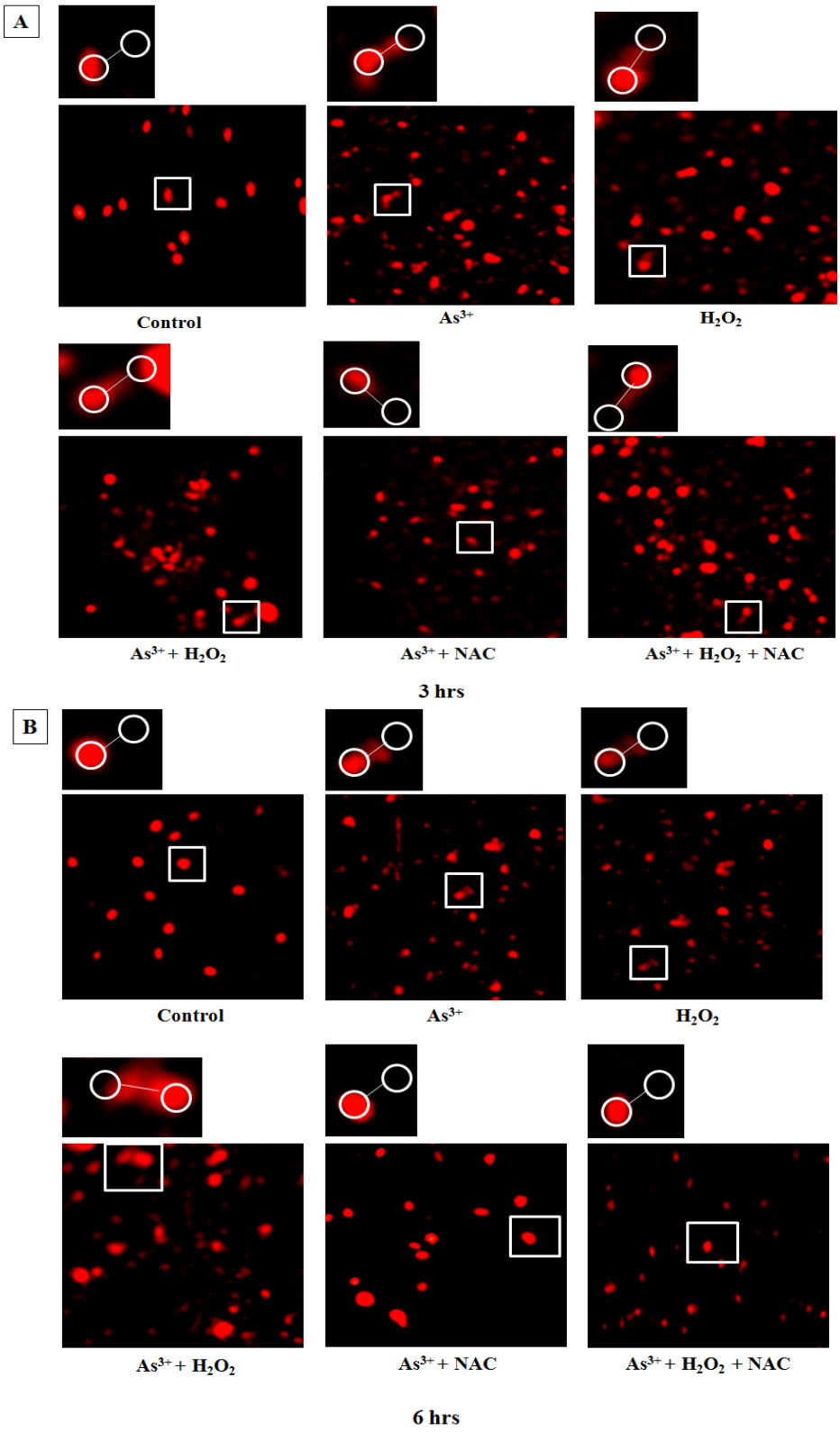

Fig. 5.5A & B: Outcome of NAC in arsenic and H_2O_2 propagated single cell degradation and tail diameter of degraded cell. The time dependent functioning of

NAC with respect to As^{3+} and H_2O_2 on hepatic single cell damage. NAC exhibits its function as a shield against these toxic agents and compensated the damage.

Table 5.2

	Comet in number		Comet tail length (µm)	
Groups	**3 hrs**	**6 hrs**	**3 hrs**	**6 hrs**
Control	1.63±0.2	1.75±0.17	6.82±0.54	5.86±0.76
As^{3+} (0.6 ppm)	6.83±0.31**	11.58±1.8***	35.52±2.57***	41.62±2.17***
H_2O_2 (100 mM)	6.49±0.27**	12.72±1.42***	38.31±2.21***/##	47.74±2.43***
As^{3+} + H_2O_2	8.52±0.87**/#	15.72±2.53***/#	48.64±3.61***/###	54.22±2.87***/#
As^{3+} + NAC (100mg)	5.65±0.18	5.55±0.42##	20.26±0.48##	10.93±0.62###
As^{3+} + H_2O_2 + NAC	5.78±0.41	7.83±0.75#	24.82±1.16##	12.83±1.49###

Table 5.2: Effect of NAC with respect to arsenic provoked comet formation. The results represent Mean ± SE (Standard Error), n=6. Data were evaluated using one-way-ANOVA following Dunnett's test (post-hoc). *p<0.05, **p<0.01 and ***p<0.001 were considered for significance analysis when compared between control and As^{3+}, H_2O_2, and As^{3+} + H_2O_2 group, whereas #p<0.05 and ##p<0.01 were considered for comparison between As^{3+} and rest of additional groups.