Chapter –III

DOSE DEPENDENT EFFECT ON SIMULTANEOUS EXPOSURE OF LEAD AND CADMIUM ON OVARIAN FUNCTION.

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Introduction

Heavy metals such as lead and cadmium are important environmental contaminants and their harmful effects on human being is of great concern today. Lead and cadmium are two metal pollutants, which has shown to be accumulated in reproductive tissues (Bires et al., 1995; Kumar and Pant, 1984) and their effect is manifested in form of reproductive malfunctioning. Reproductive performance in females is dependent on the stage of the estrous cycle. Pro-estrous stage is represented by the follicogenesis where as estrous stage represented by luteinization.

Accumulation of heavy metals in the ovarian tissue causes atresia of follicles (Junaid et al., 1997; Bires et al., 1995), hemorrhage and necrosis in estrus cycling rats on cadmium exposure (Parizek, 1983). However, few reports are contradictory where no change in ovarian structure could be demonstrated (Parizek, 1983; Paksy et al., 1989). Administration of single dose of cadmium chloride (2.2 μmol/ 100g of .b. wt) caused a disturbance and prolongation in estrus cycle (Godowicz and Pawlus,1985).

Several Reports have shown that lead and cadmium interfere with steroidogenesis (Wiebe et al., 1988a; Paksy et al., 1989; Piasek and Laskey, 1994) and thus affect the hormone levels. Estrogen and progesterone levels have been shown to decrease with lead and cadmium exposure (Wiebe et al., 1988a; Piasek and Laskey, 1994; Paksy et al., 1997).

The above reports have shown that the toxicants can affect ovarian structure; steroidogenesis and hormone profile both in dose dependent and stage specific manner. Moreover, in all above studies, the dose and time of exposure of toxicant is different. However, in the environment they might exist together at very low level, which might

contribute to either additive or antagonistic effect. Doses (0.025, 0.05, 0.1 mg/ kg. body wt) used in present study are less than above studies and designed to study the effect of simultaneous exposure on the gonadal organs over a period of time. In view of this, dose dependent and time dependent effect of lead and cadmium on the key enzymes of steroidogenesis – [3 β Hydroxy Steroid Dehydrogenase (3βHSDH), 17 β Hydroxy Steroid Dehydrogenase (17 β HSDH)] , serum and ovarian steroid hormones along with ovarian morphology and distribution of metals have been evaluated. Apart from this, toxicity parameters were also analyzed.

Experimental design

The experiment was carried out dose dependent and time dependent manner.

Dose dependent study

Regime for dose dependent study: The animals were divided into four groups, control (sodium acetate), lead acetate and cadmium acetate both alone and in combination. The animals received metal solutions of different doses (0.025 mg/kg. body wt, 0.05 mg/kg. body wt and 0.1 mg/kg. body wt) intraperitonially for 15 days. The combined treated group received same dose by taking half concentration of each metal (lead and cadmium). Two separate sets of animals were used for studying the effects of lead and cadmium in both proestrous and estrous stage of the ovarian cycle. Presence of epithelial cells confirmed the proestrous stage and presence of cornified cells confirmed estrous stage of the ovarian cycle.

Proestrous stage : After 15 days of metal exposure, blood was collected from the orbital sinuses at early proestrous stage of estrous cycle for progesterone estimation and at late proestrous stage for estrogen estimation, as the hormones remains in the baseline. Serum was separated by centrifugation at 3000 rpm for 15 minutes at room temperature and stored at -20° C until estimated using RIA kits, manufactured by Immunotech, Germany. For estrogen estimation, sensitivity of assay was 3 pg/ml while intra-assay coefficient of

variation was 8.16% and inter-assay coefficient of variation was 6.18 %. For progesterone estimation, sensitivity of assay was 0.3 ng/ml while intra-assay coefficient of variation was 4.76 % and inter-assay coefficient of variation was 6.08%.

Animals were sacrificed immediately after blood collection and the proestrous ovaries were removed and processed for assessment of steroidogenic enzymes- 3βHSDH and 17 βHSDH (Shivanandappa & Venkatesh, 1997). Toxicity parameters such as serum glutamate pyruvate transaminase (Rietman and Frankel, 1957), alkaline phosphatase (Bowers & McComb, 1975), creatinine (Bonses and Taussky, 1945) and hemoglobin (Drabkin and Austin, 1932) were also assessed.

Estrous stage: Blood was collected at estrous stage after 15 days of metal exposure and serum was separated. Animals were sacrificed, ovaries were excised out and processed for estimation of steroidogenic enzymes- 3βHSDH and 17 βHSDH (Shivanandappa & Venkatesh, 1997). Serum samples were estimated for toxicity parameters as mentioned above.

Both ovaries and blood were also analyzed for lead and cadmium levels by GBC 902 Atomic Absorption Spectrophotometer. Histology of ovary and uterus, in both stages was done using standard histological techniques.

Regime for time dependent study: In dose dependent study, most of the parameters did not demonstrate any stage specific differences. Hence, time dependent study was performed only in proestrous rats considering the fact that proestrous is important stage for follicular development and ovulation. The optimum dose selected for further experiments was 0.05 mg/kg. body wt for 30 and 45 days with four group of animals – control (sodium acetate), lead acetate, cadmium acetate and lead and cadmium acetate in combination. Animals of all groups were sacrificed after metal exposure in proestrous stage, blood was collected and serum samples were obtained after centrifugation at 3000 rpm for 15 min, and were used for

analysis of various toxicity parameters. The ovaries, uterus were immediately excised out and assessed for steroidogenic enzymes (3βHSDH and 17 βHSDH). Both the tissues and blood were analyzed for lead and cadmium levels. Histology of ovary and uterus were also studied.

Results

Dose dependent studies

The animals remained healthy throughout the treatment period. The body weights (Tables 1 and 3) and ovarian weights (Tables 2 and 4) remained unaffected by the metal exposure in both stages of the estrus cycle. There was a dose dependent accumulation of lead and cadmium in blood and ovary. The accumulation of the toxicants was intermediate in combined treated groups compared to the individual treated groups (Tables 5 and 6).

The ovary sections (plate 1) showed dose dependent effect in proestrous stage. Animals receiving cadmium alone and combined treatment demonstrated fibrosis, along with less number of growing follicles in proestrous stages. This becomes more prominent as the dose increased from 0.025 mg/ kg. body wt to 0.1 mg/ kg body wt. Lead treated animals at a dose of 0.1 mg/ kg. body wt exhibited fibrotic changes while lower doses did not show much change. In estrous stage, ovaries did not demonstrate any change.

The uteri sections (Plate 2) at demonstrated no change from the control at a doses of 0.025, 0.05 mg/kg .body wt. in proestrous stage while highest dose of 0.1 mg / kg. body wt exhibited a change in endometrial glands in all metal treated groups compared to control. In the estrus stage, animals treated with cadmium alone and with both lead and cadmium in combination showed fibrosis, along with atrophy of glands. This effect was intensified with increase in dose (Plate 3).

Figures 1and 2 explains the effect of various doses on 3β HSDH and 17 βHSDH activities in the proestrus stage of estrus cycle. The inhibitions of the enzymes were seen in a

dose dependent manner from 0.025 to 0.05 mg/ kg. b. wt, but no significant inhibition was seen as dose of the metal treatment increased from 0.05 mg/kg. body wt to 0.1 mg/kg. body wt. The pattern of inhibition for both the enzymes of steroidogenesis in estrous stage was similar to that seen in proestrous stage (Figures 3 and 4), where cadmium treated animals showed maximum inhibition and animals exposed to lead exhibited minimum inhibition.

On the basis of the inhibitory pattern of steroidogenic enzyme, steroid hormones – estrogen and progesterone were analyzed only in animals treated with metals at a dose of 0.05 mg/kg. body wt for 15 days. Both hormones showed decrease upon metal treatment (Table 7).

Toxicity parameters in both proestrous stage and estrous stage are represented in Tables 8, 9. Liver function tests like serum GPT and serum ALP and kidney function test like serum creatinine were done to evaluate the toxic effect of administered dose of metal solution. All metal treated groups showed a dose dependent decrease in serum ALP activity, along with a increase in SGPT activity. Serum creatinine levels were increased in both individually and combined treated groups, but results were within the normal range. No change in hemoglobin levels was found after the metal exposure.

Table 1: Dose dependent effect of lead and cadmium treatment for 15 days on body weight (g) in proestrus stage

Groups	0.025 mg/kg b wt		0.05 mg/kg b wt		0.1 mg/kg b wt	
	Before Treatment	After Treatment	Before Treatment	After Treatment	Before Treatment	After Treatment
Control (NaAc)	180.2 ± 7.9	202.2 ± 4.8	184.5± 6.3	195.6 ± 4.5	186.2 ±2.8	198 ± 4.8
Lead	182.2±6.98	198.8± 6.9	187.4 ± 6.5	200 ± 5.88	195 ± 4.89	200.4 ±4.4
Cadmium	184.6 ± 4.2	195.6±5.55	191.4 ± 8.2	213.6 ± 9.3	189 ±5.4	200± 6.6
Lead + Cadmium	187.1±4.94	199.6 ± 4.9	190 ± 3.8	205.6 ± 7.8	180.4± 5.7	201 ± 3.8

Table 2: Dose dependent effect of lead and cadmium treatment for 15 days on ovarian weight (mg) in proestrous stage

Groups	0.025 mg/kg b wt	0.05 mg/kg b wt	0.1 mg/kg b wt
Control (NaAc)	111 ± 3.8	116.8 ±2.9	105 ± 4.3
Lead	120.8 ± 2.65	108.8 ±2.09	100 ± 4.93
Cadmium	102.2 ±5.52	99.8 ±2.8	83.4 ±5.06
Lead + Cadmium	100 ±3.9	108.9 ±5.9	99.4 ± 3.9

Table 3: Dose dependent effect of lead and cadmium treatment for 15 days on body weight (g) in estrous stage

Groups	0.025 mg/kg b wt		0.05 mg/kg b wt		0.1 mg/kg b wt	
	Before Treatment	After Treatment	Before Treatment	After Treatment	Before Treatment	After Treatment
Control (NaAc)	184.2± 3.9	197.2 ± 3.8	189.8± 5.3	197.6 ± 2.5	186.2± 2.7	196 ±3.8
Lead	188.2 ±5.9	197.8 10.9	189.4 ±4.5	200 ±7.88	192 ± 2.89	202.4 ±2.4
Cadmium	186.6 ±5.2	185.6 ± 6.5	201.4± 4.2	203.6± 10	189 ± 3.4	199± 3.59
Lead+ Cadmium	185 ± 5.9	189.6 ±6.9	200 ± 3.8	205.6± 3.8	190.4± 5.7	200 ± 4.7

Table 4: Dose dependent effect of lead and cadmium treatment for 15 days on ovarian weight (mg) in estrus stage

Groups	0.025 mg/kg b wt	0.05 mg/kg b wt	0.1 mg/kg b wt
Control (NaAc)	102 ±2.3	101.6 ± 3.7	106 ± 3.3
Lead	95.7 ± 4.5	101.4 ±1.69	95.2 ± 2.5
Cadmium	98.2 ± 2.9	85.8 ± 3.9	83.2 ± 3.84
Lead + Cadmium	99.2 ± 3.9	97.6 ± 2.6	89 ± 3

Table 5: Dose Dependent effect of lead and cadmium alone and in combination on blood lead and cadmium content (μg/ml)

Dose	0.025 mg/kg b wt		0.05 mg/kg b wt		0.1 mg/ kg b wt	
	Lead	**Cadmium**	**Lead**	**Cadmium**	**Lead**	**Cadmium**
Con	N.D	N.D	N.D	N.D	N.D	N.D
Pb	1.83 ±0.10 [a]	N.D.	3.92 ±0.06 a,**	N.D.	5.8 ±0.27 a,**, ##	N.D.
Cd	N.D.	0.076±0.01 [a,l]	N.D.	0.205 +0.035 a,l,**	N.D.	0.299 ±0.049 a,l,**, ##
Pb+ Cd	1.21±0.4 [a,l]	0.055±0.02 [a,l,f]	1.54 ±0.09 a,l,**	0.13±0.04 a,l,**, d, ##	3.2±0.27 a,l,**, ##	0.159 ±0.08 a,l,d,**, ##

N.D.= Not Detectable, n=3-4. The values are mean ± SEM.

[a] P <0.001compared to control of identical dose; [l] P<0.001compared to lead of identical dose ; [d] P <0.001, [f] P <0.01 compared to cadmium of identical dose; ** P<0.001 within same metal treatment compared to be lowest dose; [##] P <0.001 within the same metal group compared to 0.05 dose .

Table 6: Dose Dependent effect of lead and cadmium alone and in combination on ovarian lead and cadmium content (ng/ mg wet weight)

Dose	0.025 mg/kg b wt		0.05 mg/kg b wt		0.1 mg/ kg b wt	
	Lead	Cadmium	Lead	Cadmium	Lead	Cadmium
Con	N.D	N.D	N.D	N.D	N.D	N.D
Pb	1.47± 1.01 [a]	N.D	3.14± 0.24 a,**	N.D	6.06± 0.42 [a,] **, ##	N.D
Cd	N.D	1.65±0.01 [a,1]	N.D.	3.52 ±0.01 [a,1,#]	N.D	4.43±0.03 a,##,1
Pb+ Cd	0.78 ± 0.33 c,1	0.79 ± 0.08 b,2,d	1.53±0.26 a,1,*	2.27± 0.03 a,f,1,**	4.33±0.18 a,1,d,**,##	2.73±0.02 a,1,**,#

N.D. = Not detectable

n=3-4. The values are mean ±SEM.

[a] P<0.001; [b] P<0.01;[c] P<0.05 compared to control of identical dose; [1] P <0.001; [2] P<0.05 compared to lead acetate of identical dose; [d] P<0.001; [f] P<0.01 compared to cadmium acetate of identical dose; ** P<0.001; *P<0.05 within same metal treatment compared to be lowest dose; [##] P<0.001 ,[#] P<0.05 within the same metal group compared to 0.05 dose .

Plate 1: Histological observation of ovary after various doses (0.025, 0.05 and 0.1mg/kg.body wt) of lead and cadmium treatment.

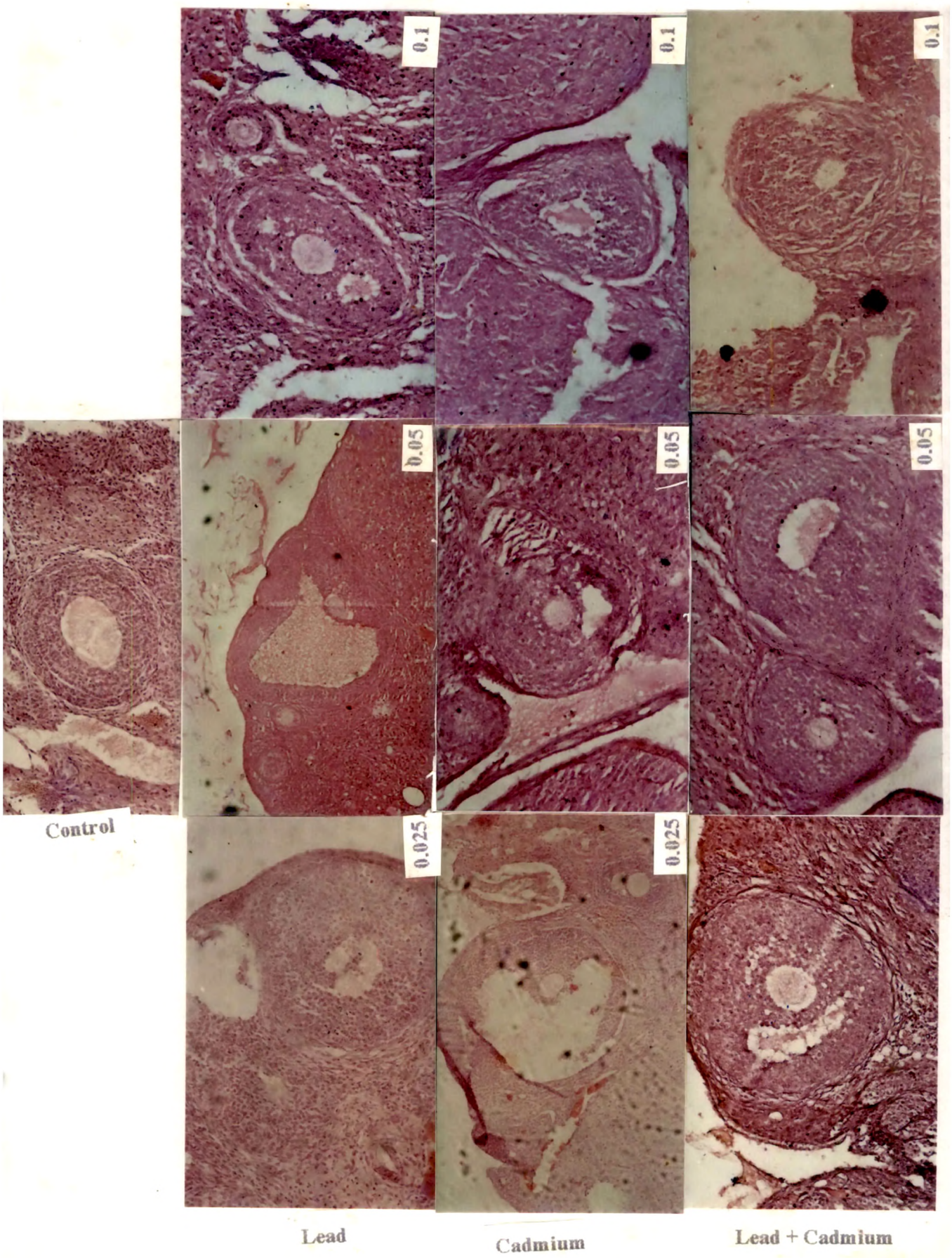


Plate 2: : Histological observation of uterus after various doses (0.025, 0.05 and 0.1mg/kg.body wt) of lead and cadmium treatment in proestrous stage.

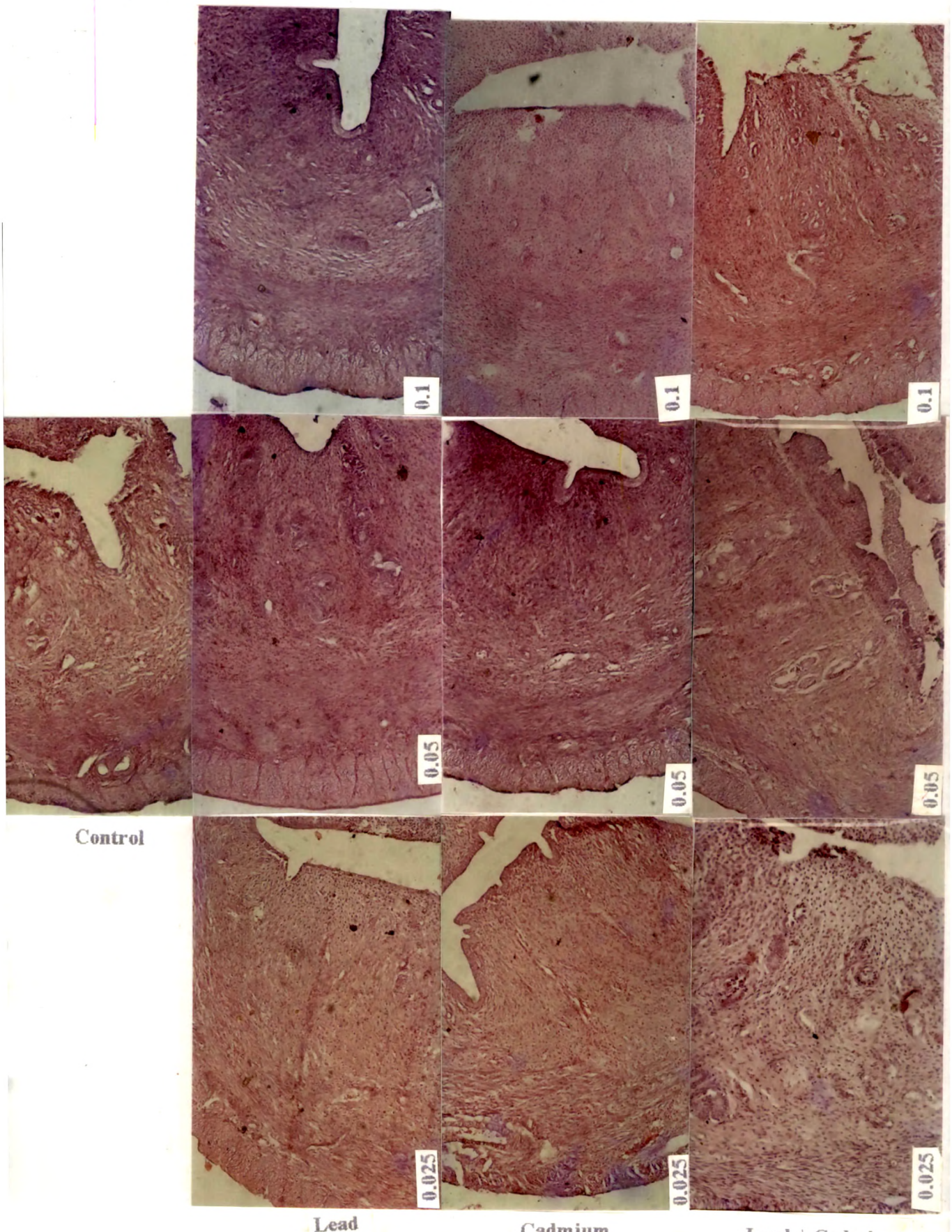


Plate 3: Histological observation of the uterus after various doses (0.025, 0.05 and 0.1mg/kg.body wt) of lead and cadmium treatment at estrous stage.

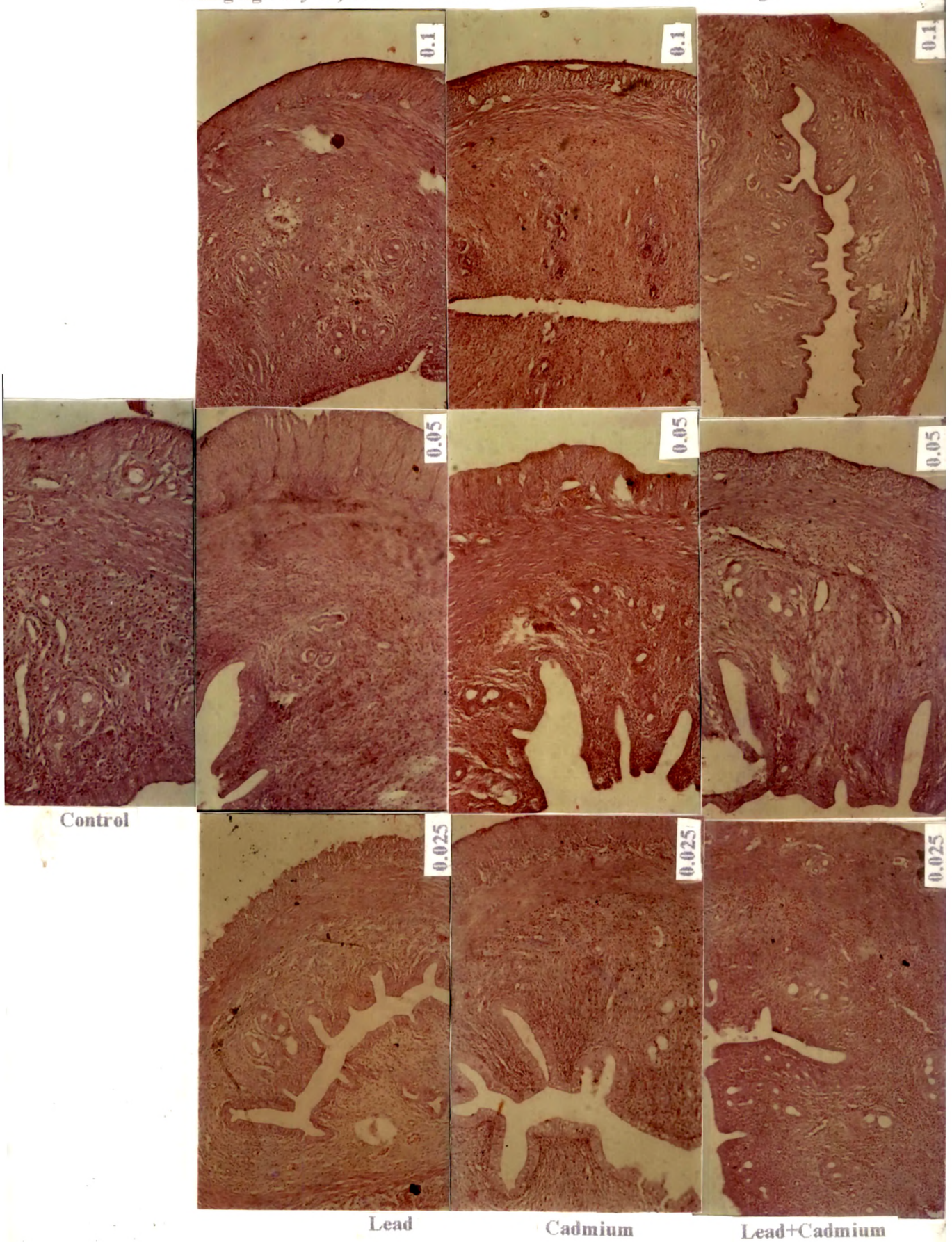
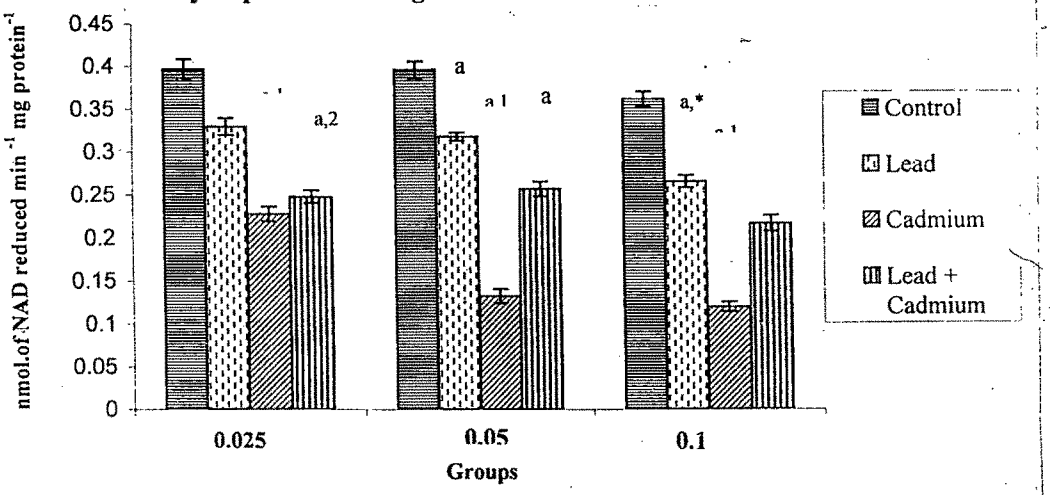


Fig 1: Dose dependent effect of lead and cadmium either alone or in combination on ovarian 3 β Hydroxy Steriod Dehydrogenase (3βHSDH) activity in proestrous stage.



N=6 . The values are Mean ± SEM.

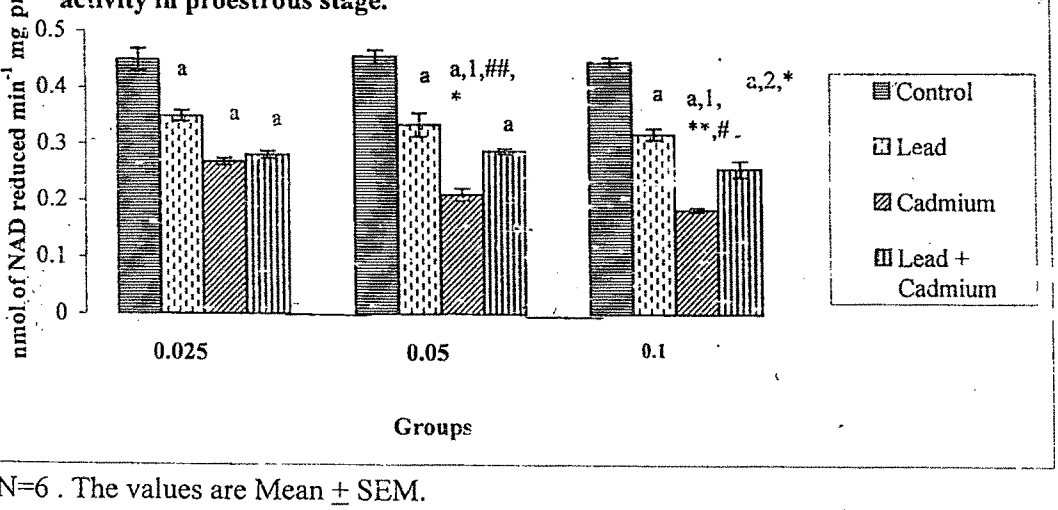
a P<0.001 , b P<0.01 compared to control of identical dose.,

P 1<0.001, 2<0.01 compared to lead group of identical dose.

P <0.001 compared to lead + cadmium group of identical dose

*** P<0.001, * P<0.05 compared within the group to the lowest dose.

Fig 2: Dose dependent effect of lead and cadmium either alone or in combination on ovarian17 β Hydroxy steriod Dehydrogenase (17 β SHDH) activity in proestrous stage.



N=6 . The values are Mean ± SEM.

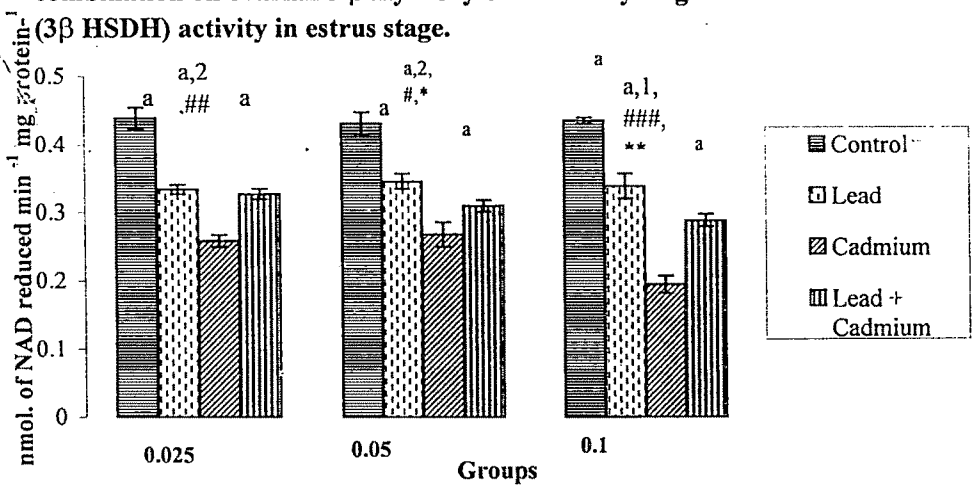
a P<0.001 compared to control of identical dose.,

1 P<0.001, 2 P<0.01 compared to lead group of identical dose.

P<0.001,# P<0.01 compared to lead + cadmium group of identical dose

*** P<0.001,*P<0.05 compared within the group to the lowest dose.

Fig 3: Dose dependent effect of lead and cadmium either alone or in combination on ovarian 3 β Hydroxy Steroid dehydrogenase (3β HSDH) activity in estrus stage.



N=6 . The values are Mean ± SEM.

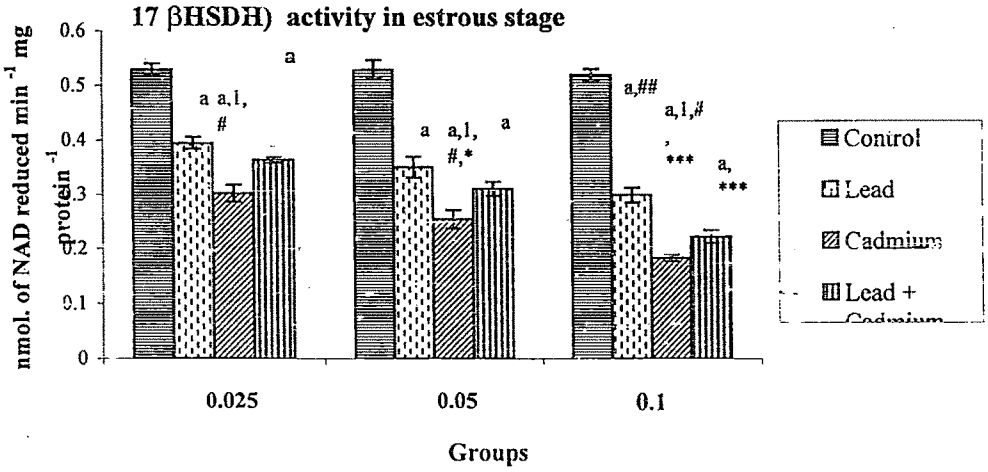
a P<0.001 compared to control of identical dose.,

1 P<0.001, 2 P<0.01 compared to lead group of identical dose.

P<0.001, ## P<0.01,#P<0.05 compared to lead + cadmium group of identical dose

** P<0.01,* P<0.05 compared within the group to the lowest dose.

Fig 4: Dose dependent effect of Lead and Cadmium alone and in combination on ovarian 17 β Hydroxy Steroid Dehydrogenase (17 βHSDH) activity in estrous stage



N=6 . The values are Mean ± SEM.

a P<0.001 compared to control of identical dose.,

1 P<0.001 compared to lead group of identical dose.

P<0.01,# P<0.05 compared to lead + cadmium group of identical dose

*** P<0.001,* P<0.05 compared within the group to the lowest dose.

Table 7: Effect of lead and cadmium either alone or in combination on gonadal steroids.

GROUPS	Serum Estrogen (pg./ml)	Ovarian Estrogen (pg./mg ovary)	Serum Progesterone (ng/ml)	Ovarian progesterone (ng/mg ovary)
Control	81.57 ± 4.88	18.3 ±2.27	80.75 ± 5.46	0.212 ±0.017
Lead	61.25 ± 6.22 [a]	9.55 ± 0.456 [a]	67.87 ±3.72	0.098 ±.0035 [b]
Cadmium	44.5 ± 2.06 [a,2]	7.0 ± 0.090 [a]	33.5 ±5.35 [a,2]	0.071 ±.0019 [b]
Lead + Cadmium	50.38 + 1.19 [b,1]	8.02 ±0.189 [a]	47.5 ± 4.19 [b,1]	0.093 ±.001 [c]

N=4. The values are Mean ±SEM.

[a] P<.001, [b] P< 0.01, [c] P<0.05 compared to control

[1] P<0.05, [2] P<0.01 compared to lead group

Table 8: Dose dependent effect of lead and cadmium on safety parameters in proestrous stage.

Groups	Dose (mg/kg b wt)	Parameters			
		Hemoglobin (g%)	SGPT (IU/dl)	ALP (IU/dl)	Serum creatinine (mg%)
Control (NaAc)	0.025	14.32 ± 0.81	23.4 ±2.06	101.6 ±6.7	0.84 ± 0.05
	0.05	15.62 ±1.4	25.6 ± 1.85	102.8 ±9.1	0.79 ± 0.09
	0.1	15.62 ± 0.54	24.7 ±1.6	101.0 ±5.2	0.89 ± 0.08
Lead	0.025	14.72 ±0.24	26.2 ±1.7	79 ± 2.54	0.86 ± 0.09
	0.05	15.2 ±0.47	29.4 ± 0.9	69.8 ±0.8	0.83 ±0.11
	0.1	15.3± 0.27	29.5 ± 1.4	61.3 ± 2.3	1.02 ±0.08
Cadmium	0.025	14.87 ± 0.73	30.4 ± 1.4	60.5 ±1.81	1 ± 0.06
	0.05	14.9 ± 0.53	34.6 ± 2.0	59.7 ± 1.57	0.96 ± 0.12
	0.1	15.1 ± 0.27	41.5 ± 2.7	50.6±1.97	1.4 ± 0.134
Lead + Cadmium	0.025	15.0 ± 0.3	23.3 ± 1.3	70.1 ± 2.3	1± 0.063
	0.05	14.9± 0.24	29.8 ± 1.4	66.4 ± 2.2	1.0 ± 0.07
	0.1	15.2 ± 0.31	39.8 ±1.8	61.9 ± 2.15	1.2 ± 0.108

N=5-6. The values are Mean ±SEM.

ALP: IU- μ moles of PNP formed/ min

SGPT: IU-μ moles of pyruvate formed/ min

Table 9: Dose dependent effect of lead and cadmium on safety parameters in estrus stage.

Groups	Dose (mg/kg b wt)	Parameters			
		Hemoglobin (g%)	SGPT (IU/dl)	ALP (IU/dl)	Serum creatinine (mg%)
Control (NaAc)	0.025	15.5 +0.34	22 +1.4	100.7 + 5.02	0.80 +0.05
	0.05	15.5 +0.164	22.9 +0.94	98 +3.5	0.9 +0.04
	0.1	14.6 +0.564	27.6 +18	103.2 + 4.1	0.92 + 0.03
Lead	0.025	14.77 +0.54	23.2 +1.34	76.3 +2.09	0.8 +0.05
	0.05	14.4 + 0.33	22.7 +0.62	72 +3.6	1.08 +0.031
	0.1	14.9 +0.23	29.6 +1.11	62.7 +2.2	0.9 +0.04
Cadmium	0.025	15.0 +0.37	28.2 +1.83	60.3 +1.5	0.92 +0.05
	0.05	15.4 +0.237	29.4 +1.96	59.8 +2.7	1.2 +0.02
	0.1	15.23 +0.25	31.6 +1.8	56.8 +3.8	1.3 +0.04
Lead + Cadmium	0.025	15.2 +0.28	22.7 +2.9	70.9 +2.5	0.83 +0.04
	0.05	15.6 +0.27	28.3 +2.0	69.3 +2.23	1.09 +0.05
	0.1	15.3 +0.27	32.6 +0.84	61.4 +2.3	1.1 +0.04

n=5-6. The values are Mean ±SEM.

ALP: IU- μ moles of PNP formed/ min

SGPT: IU-μ moles of pyruvate formed/ min

Time dependent studies

Exposure to metal for different time period did not affect the body weights and ovarian weights (Tables 10 and 11). Tables 12-14 represents the results of metal content in ovary, uterus and blood respectively, of animals exposed to metals for different time periods. There was progressive increase in uterine, ovarian and blood content of lead and cadmium with increase in time of exposure.

Ovarian 3β HSDH and 17β HSDH activities were further inhibited in lead and combined treated groups with increase in time of exposure from 15 days to 30 days. However, the inhibition was not significantly different when compared between 30 and 45 days of exposure in combined treated group. In case of cadmium treated group, further decrease in 3β HSDH activity could not be demonstrated with increase in time of exposure of metals. Although, 17β HSDH activity did show time dependent decrease in cadmium treated group (Figures 5 and 6).

Ovary sections (Plate 4) demonstrates time dependent effect of lead, cadmium in isolation and in combination. Animals receiving lead acetate for 30 days did not show much change from control, while cadmium exposure resulted in a decrease in follicle number, shrunken cells and an increase in atretic follicles. Animals receiving both lead and cadmium demonstrated an increase in interstitial fibrosis, a decrease in number of follicles with atrophy. This effect was pronounced as time of exposure increased to 45 days.

Time dependent effect of lead and cadmium on uterus is represented in plate 5. Uteri of 15 and 30 days lead and cadmium treated groups exhibited no change from the control while exposure to metals for 45 days resulted in a structural change. Lead treatment for 45 days caused a decrease and atrophy in endometrial glands while cadmium and combined treated groups exhibited a decrease in endometrial glands and shedding of endometrial epithelium.

Table 10: Time dependent effect of lead and cadmium treatment on body weight (g) in proestrous stage

Groups	15 Days		30 Days		45 Days	
	Before Treatment	After Treatment	Before Treatment	After Treatment	Before Treatment	After Treatment
Control (NaAc)	180.2 ± 7.9	202.2 ± 4.8	182.4 ±3.3	201.2 ± 2.2	184.6 ±4.3	208 ±5.12
Lead	182.2 ± 6.9	198.8 ± 6.9	182 ± 4.9	203.2 ±5.2	191.2 ± 3.3	210 ±5.5
Cadmium	184.6 ± 4.2	195.6 ± 5.5	189.4± 4.5	200.8 ±5.4	184.4±3.9	198.4± 3.7
Lead + Cadmium	187.1± 4.9	199.6 ± 4.9	187.2 ±3.1	199.4 ±3.6	186 ±7.6	211± 6.9

Table 11: Time dependent effect of lead and cadmium treatment on ovarian weight (mg) in proestrus stage

Groups	15 days	30 days	45 days
Control (NaAc)	116.8 ±2.9	111 ± 9.86	108.9 ± 3.6
Lead	108.8 ±2.09	102 ± 4.6	99.8 ± 3.4
Cadmium	99.8 ±2.8	103 ± 3.1	97.6 ± 4.5
Lead + Cadmium	105.9 ±5.9	101.6 ± 6.1	100.1 ± 3.3

Table 12: Time Dependent effect of lead and cadmium either alone or in combination on blood lead and cadmium content (μg/ml) at a dose of 0.05 mg/kg .body .wt.

Dose	15 days		30 days		45 days	
	Lead	Cadmium	Lead	Cadmium	Lead	Cadmium
Con	N.D	N.D	N.D	N.D	N.D	N.D
Pb	3.92 ±0.06 a	N.D.	4.26 ±0.54 a	N.D.	5.7±0.44 a,**,##	N.D
Cd	N.D.	0.21 ±0.04 e	0.81±0.2	0.234 ±0.013 a	1.1 ±0.07	0.4±0.03 a,**,##
Pb+ Cd	1.54±0.09 a	0.13±0.04 e	1.6±0.13 a,I	0.19 ±0.014 a	1.7 ±0.16 a,I	0.34±0.04 a,**,##

N=3-4. The values are mean ± SEM. [a] P<0.001 compared to control; [I] P<0.001 compared to lead and [e] P<0.001 compared to cadmium of similar treatment regime; ** P<0.001 and ## P<0.001 compared to 15 and 30 days respectively.

Table 13: Time Dependent effect of lead and cadmium alone and in combination on ovarian lead and cadmium content (ng/mg wet weight) at a dose of 0.05 mg/kg.b.wt.

Dose	15 days		30 days		45 days	
	Lead	Cadmium	Lead	Cadmium	Lead	Cadmium
Con	N.D	N.D	0.8 + 0.02	0.67 ± 0.01	0.91 ± 0.04	0. 77 ±0.02
Pb	3.13± 2.4 a	N.D	5.2 + 0.11 a,###,***	0.71 +0.03	7.12 ±0.14 a, ###,***,ĀAA	0.80 ±0.05
Cd	N.D.	3.5 ±0.001 a,I	0.80 ± 0.03	5.8 ±0.18 a, I,***	0.92 ±0.02	6.9 + 0.21 a, I,***,AĀA
Pb+ Cd	1.53 ± 2.6 a,i	2.2± 0.003 a,I	3.2±0.08 a,I,###,***	4.5 ±0.17 a, I, ###	4.4 ±0.21 a,I,###,***,ĀAA	5.3 ±0.11 a, I,###,***,AAA

N= 3-4. the values are Mean ± SEM.
A p<0.001 compared to control group;[I] p<0.001 compared to lead group
[###] p<0.001 compared to cadmium group;[***] p<0.001 compared to lowest time period of identical metal treatment; [AAA] p<0.001 compared to 30 day period of identical metal treatment

Table 14: Time Dependent effect of lead and cadmium alone and in combination on uterine lead and cadmium content (μg/g wet weight) at a dose of 0.05 mg/kg.b.wt.

Dose	15 days		30 days		45 days	
	Lead	**Cadmium**	**Lead**	**Cadmium**	**Lead**	**Cadmium**
Con	1.07 ±0.05	0.26 ±0.03	1.09 ±0.06	0.27 ± 0.01	1.1 ±0.09	0.3 ±0.15
Pb	1.9 ± 0.07 a,###	0.27 ±0.03	3.5 ± 0.12 a,###,***	0.28 ±0.026	5.3 ± 0.13 a,###,***, AAA	0.29 ±0.04
Cd	0.96 +0.02	2.03 ± 0.14 a,1	1.03± 0.01	4.18 ±0.08 a, 1,***	1.3 ±0.14	6 ±0.14 a, 1,***,AAA
Pb+ Cd	1.6 ±0.08 a,2,###	0.96 ±0.03 a,1,###	2.3 ± 0.24 a,1,###,***	2.93 ±0.14 a, 1, ###,***	3.6 ± 0.08 a,1,###,***,AAA	4.4 ±0.23 a,1,###,***,AAA

N= 3-4. the values are Mean + SEM.
a $p<0.001$ compared to control group

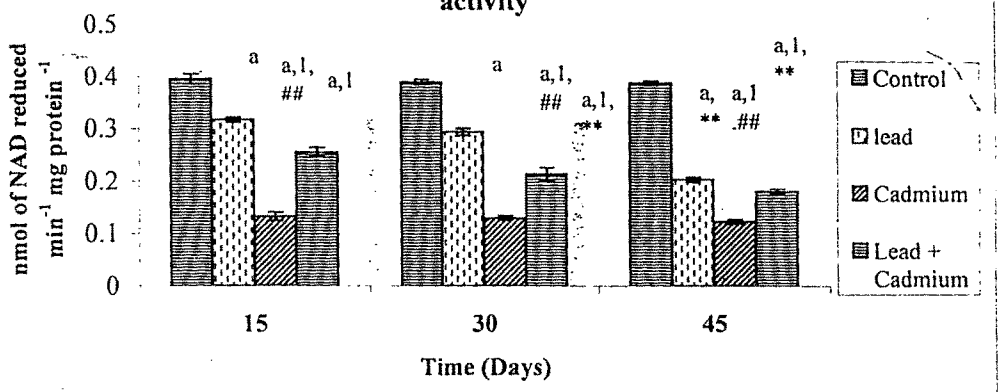
1 $p<0.001$, 2 $p<0.01$ compared to lead group

$p<0.001$ compared to cadmium group

*** $p<0.001$ compared to lowest time period of identical metal treatment

AAA $p<0.001$ compared to 30 day period of identical metal treatment

Fig 5: Time dependent effect of lead and cadmium either alone or in combination on ovarian 3 β Hydroxy steriod Dehydrogenase activity



N=6 . The values are Mean ± SEM.

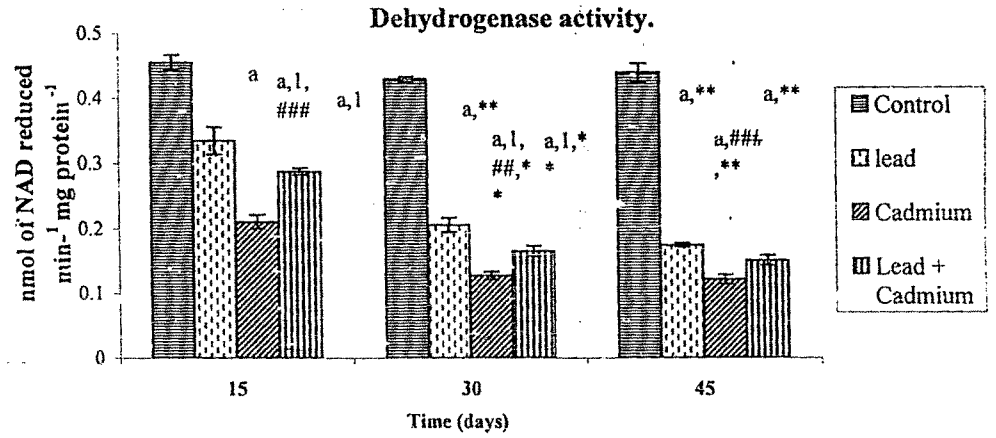
a P<0.001 compared to control of similar treatment period.

P 1<0.001compared to lead group of similar treatment period.

P <0.001 compared to lead + cadmium group of similar treatment period.

** P<0.001 compared within the group to the 15 day treatment

Fig 6: Time dependent effect of lead and cadmium either alone or in combination on ovarian 17 β Hydroxy steriod Dehydrogenase activity.



N=6 . The values are Mean ± SEM.

a P<0.001 compared to control of similar treatment period.

1 p<0.001compared to lead group of similar treatment period.

P <0.001, ## P<0.01compared to lead + cadmium group of similar treatment p

** P<0.001 compared within the group to the 15 day treatment

Plate 4: Histological observation of ovary after various time exposure (15, 30 and 45 days) of lead and cadmium treatment .

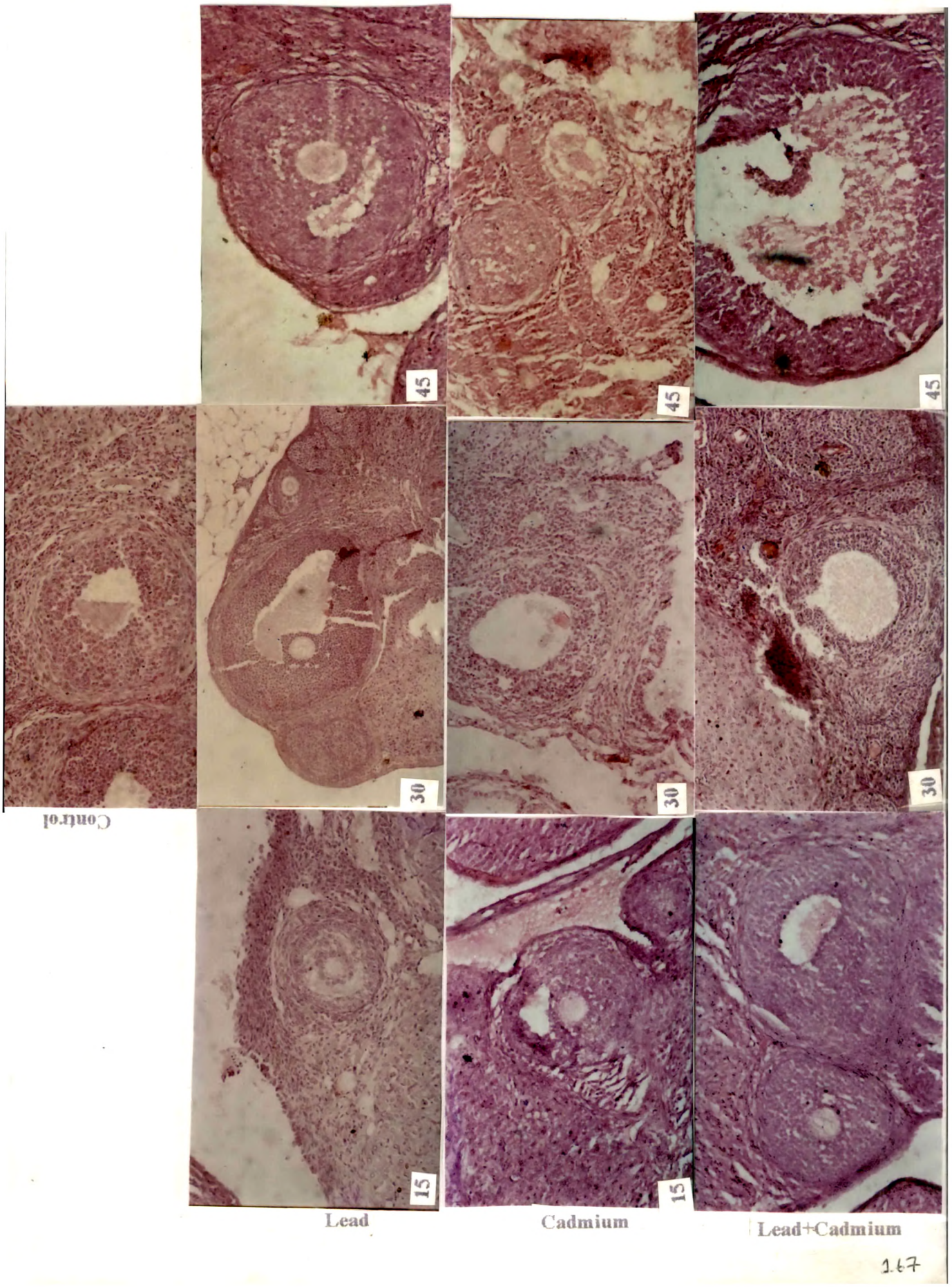
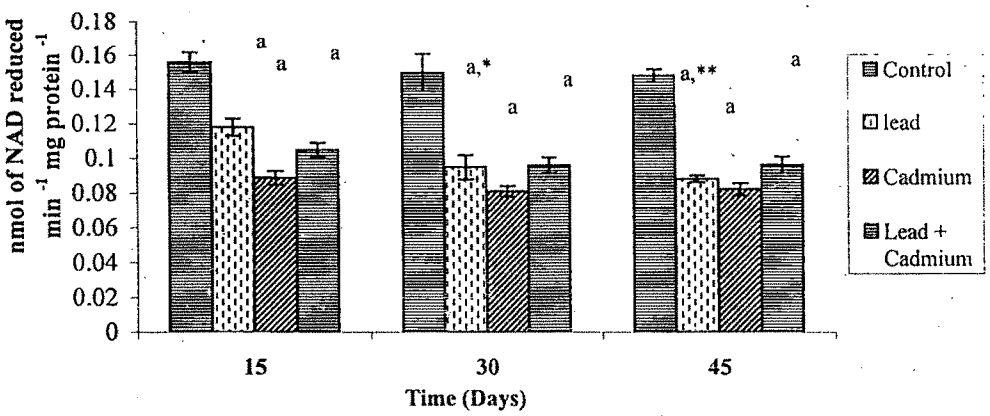


Fig 7: Time dependent effect of lead and cadmium either alone or in combination on uterine 3 β Hydroxy steriod Dehydrogenase(3 βHSDH)

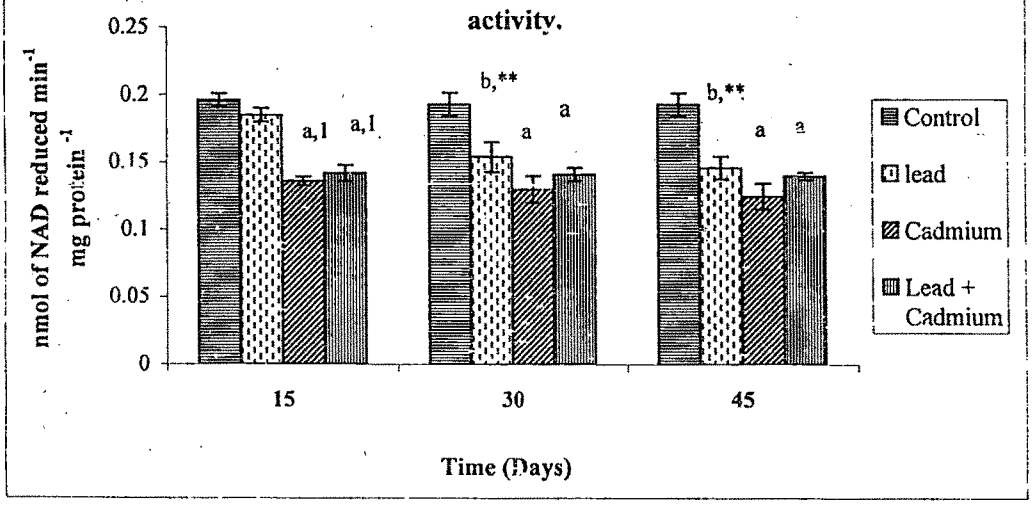


N=6 . The values are Mean ± SEM.

a P<0.001 compared to control of similar treatment period.

** P<0.01, * P<0.05 compared within the group to the 15 day treatment

Fig 8 : Time dependent effect of lead and cadmium either alone or in combination on uterine 17 β Hydroxy steriod Dehydrogenase activity.



N=6 . The values are Mean ± SEM.

a P<0.001,b P<0.01 compared to control of similar treatment period.

1P<0.001 compared to lead group of similar treatment period

** P<0.001 compared within the group to the 15 day treatment

Plate 5: Histological observation of uterus after various time exposure (15, 30 and 45 days) of lead and cadmium treatment .

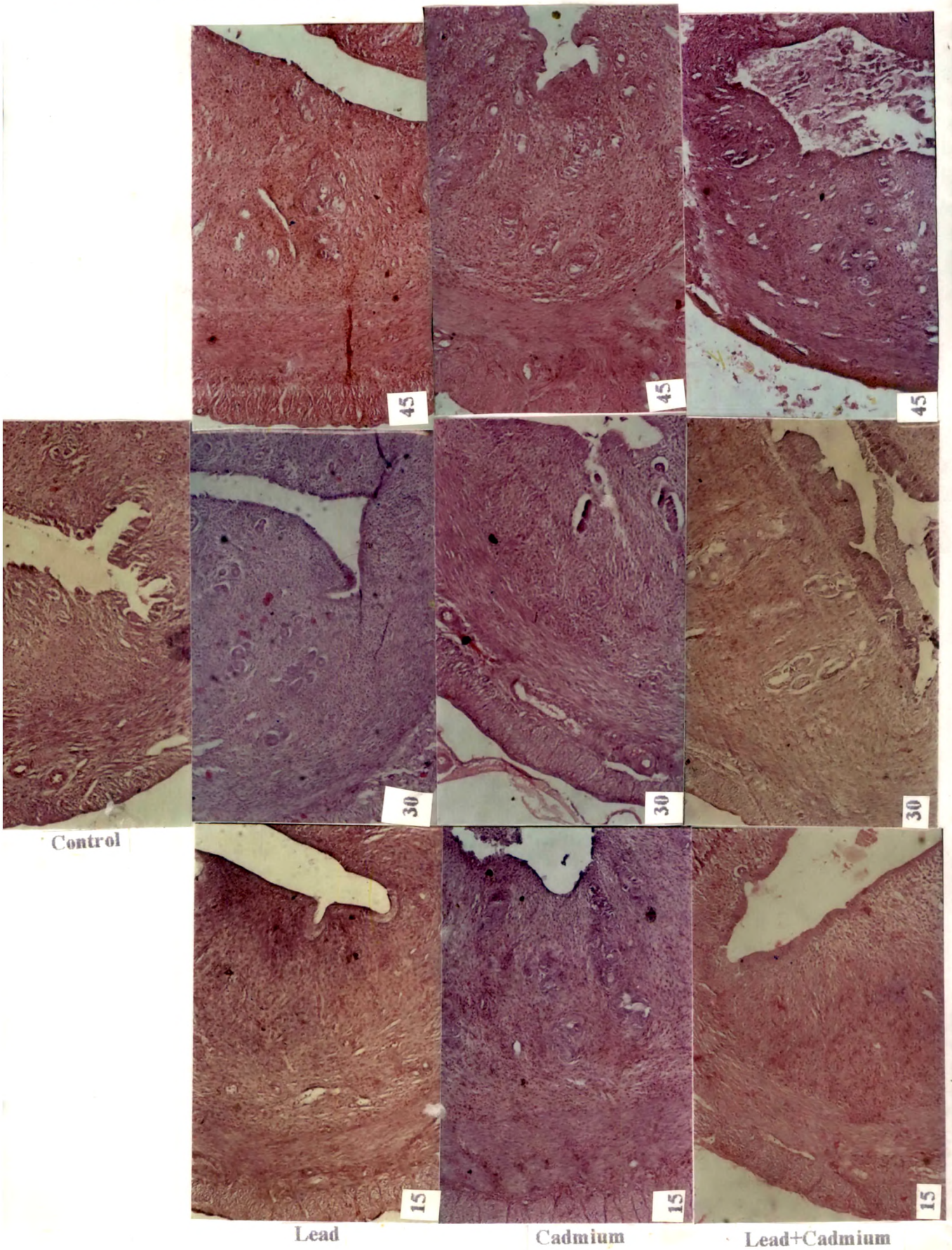
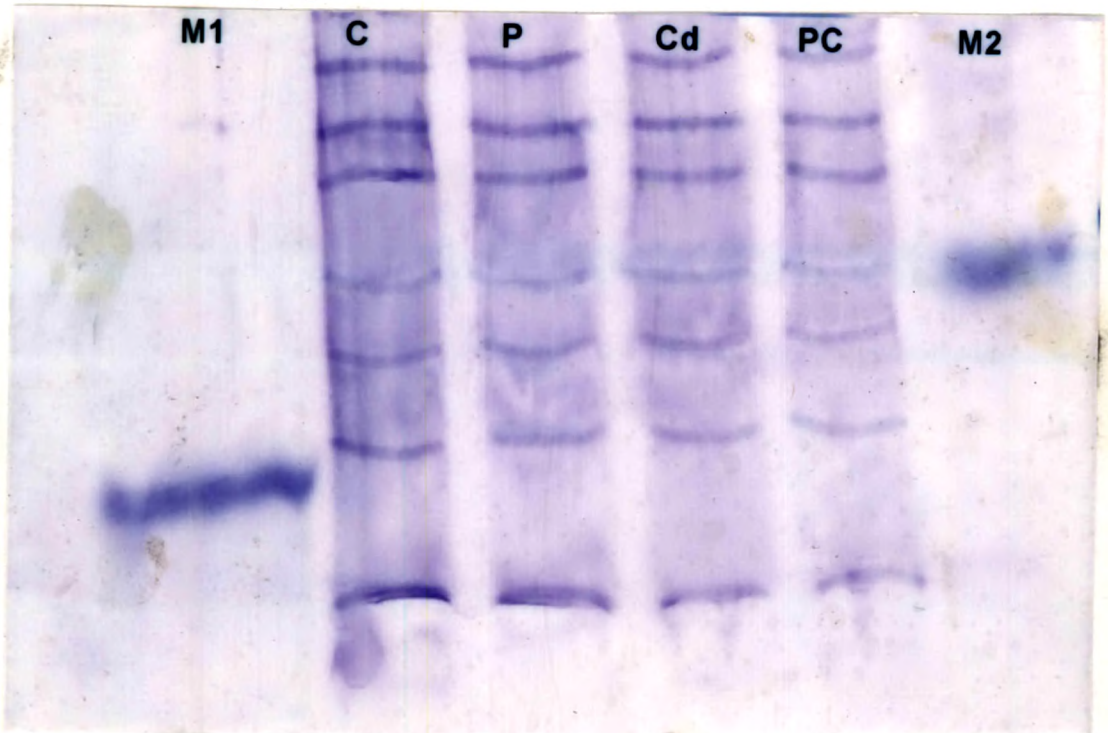
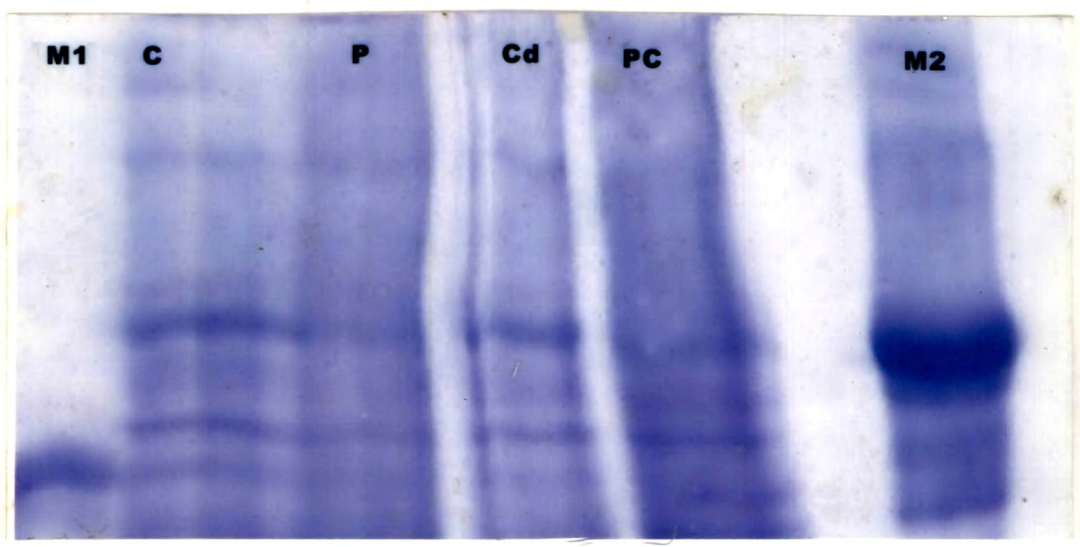


Plate 6: Electrophoretic Protein profile of uterus after lead and cadmium exposure at different time of exposure(15, 30 and 45 days)

a) After 15 day exposure

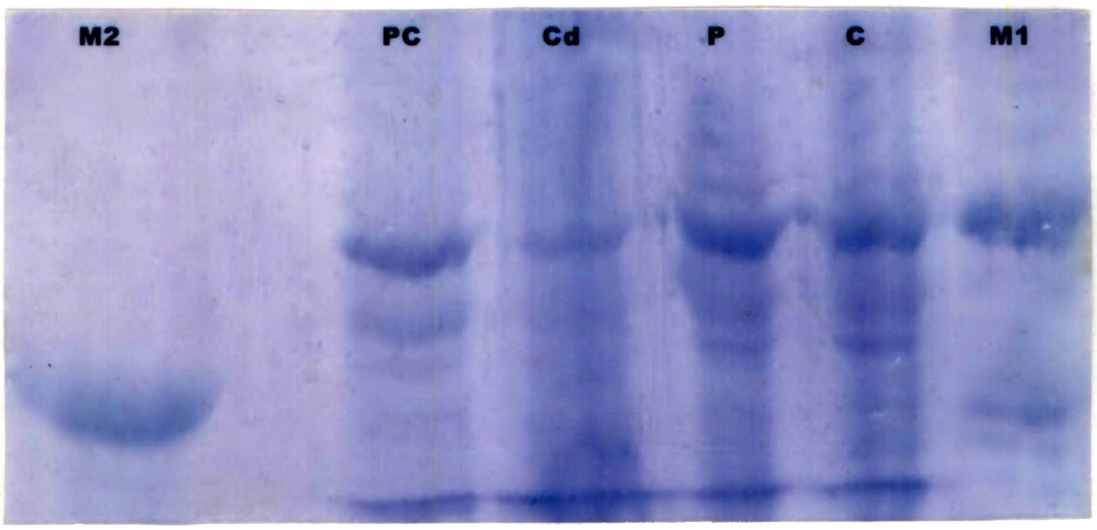


b)After 30 day of exposure



M1- pepsin (34 kDa), M2- BSA(66 kDa), C-Control, P-Lead, Cd-cadmium and PC-lead+ cadmium

b) After 45 day of exposure



M1- BSA(66 kDa), M2- pepsin (34 kDa), C-Control, P-Lead, Cd-cadmium and PC-lead+ cadmium

Table 15: Time dependent effect of lead and cadmium on safety parameters in proestrous stage.

Groups	Duration of treatment in days	Parameters			
		Hemoglobin (g%)	SGPT (IU/dl)	ALP (IU/dl)	Serum creatinine (mg%)
Control (NaAc)	15	15.62 ±1.4	25.6 ± 1.85	102.8 ±9.1	0.79 ± 0.09
	30	15.12 ±0.67	23.7 ±0.93	100.8 ±5.1	0.93 ±0.08
	45	15.2 ±0.72	26.3 ±0.79	100.4 +3.4	0.9 ±0.21
Lead	15	15.2 ±0.47	29.4 ± 0.9	69.8 ±0.8	0.83 ±0.11
	30	13.62 ±0.43	29.7 ±0.93	71.3 ±5.2	1.5 ±0.13
	45	14.1 ±1.2	32.3 ±1.2	67.7 ± 5.2	2.1 ±0.11
Cadmium	15	14.9 ± 0.53	34.6 ± 2.0	69.8 ±0.8	0. 96 ± 0.12
	30	14.2 ±0.42	35.4 +1.6	55.6 ±3.8	2.06 ±0.14
	45	14.1 ±0.52	34.1 +0.8	53.7 ±6.7	2.54 ±0.22
Lead + Cadmium	15	14.9± 0.24	29.8 ± 1.4	66.4 ± 2.2	1.0 ± 0.07
	30	13.3 ±0.5	29.2 +1.36	63.6 ±4.5	1.5 ±0.09
	45	13.2 ±0.32	31.7 +0.92	67.7 ±6.9	2.5 ±0.163

n=5-6. The values are Mean ±SEM.

ALP: IU- μ moles of PNP formed/ min

SGPT: IU-μ moles of pyruvate formed/ min

Uterine 3β HSDH and 17β HSDH of lead exposed animals showed time dependent inhibition but no significant change in activity of key enzymes was observed in cadmium and combined treated groups as the time of exposure increased (Figures 7 and 8).

There was no change in protein profile in uterus of all metal groups treated for 15 days. Protein profile of uterus of 30 day treated animals showed decreased expression of 30-34 kDa and 60- 66 kDa protein in lead treated animals. Expression of 30-34 kDa protein was decreased in all metal treated groups after 45 days of metal treatment. Since the quality of the gel is poor and other molecular weight markers has not been used, variation in other bands could not be commented (Plate 6).

Toxicity parameters like SGPT showed an increase as the time of metal exposure increased and serum ALP showed a decrease, all values remain within the normal range. Similarly, no significant change was observed in serum creatinine levels in the metal exposed animals compared to control. The hemoglobin levels were unaffected by the metal exposure even at 45 days of treatment. (Table 15).

Discussion

The type of hormones synthesized in a particular stage of estrus cycle defines ovarian and uterine structure. The sex steroid hormones are needed for maintenance of reproductive function. In the proestrous stage, estradiol is needed for follicular growth while progesterone is prime hormone synthesized in estrous stage in the corpus luteum. Hence, two main stages of cycle were considered for the study.

Distribution pattern shows that the toxicants-lead and cadmium get accumulated in the ovaries, uterus and blood in dose and time dependent manner. Several workers have shown the accumulation of metal salts in ovary (Bires et al., 1995; Saksena and Salmonsen, 1983). The accumulation of cadmium and lead in uterus has been reported by several

workers (Copius-Peereboom et al., 1981; Bires et al., 1995; Wiebe et al., 1988b), which is similar to our observation.

The structure of the ovary and uterus also plays an important role in the maintenance and synthesis of the steroid hormones. Cadmium induced structural changes are dependent on species, strain, age and sex of the animals. Available data in literature suggests that microcirculation in steroid sensitive reproductive organs i.e., ovary and uterus are targets for acute exposure to toxicants like Cd in rodents (Copius Peereboom-Stegeman and Jongstra-Spaapen, 1979; Di Sant' Agnese et al., 1983). Animals receiving cadmium and combined treatment at a lower dose of 0.025 mg/kg body wt also showed fibrosis, with reduced number of follicles. Similar changes were reported by several workers (Godowicz and Pawlus, 1985; Rehm and Waakles, 1988) at a doses of 22 to 47.5 μmol of cadmium /kg. Lead treated animals with 0.1 mg/ kg body wt dose also showed symptoms of fibrosis which is in accordance with observations reported by Junaid et al. (1997), where he demonstrated a lower dose of 2 mg/kg body wt of lead in dose dependent study (0-8 mg/kg body wt) was able to affect the follicles. However, inspite of structural changes, no change in estrus cyclicity was observed in the present study, which is similar to other reports (Paksy et al., 1990; Piasek and Laskey, 1994). Such structural changes may be related to apparent deficiency of the sequestering proteins like metallothionein in the ovary (Waakles et al., 1988) or related to continuos exposure of the metal ions. Long time exposure caused a change in endometrium and its epithelium. Similar structural changes are reported by Copius Peereboom-stageman (1987), where he demonstrated long term treatment caused a change in endometrial vasculature.

Every organ demonstrates structure function relationship. Structural changes observed in the present study also could be related to change in activities of key steroidogenic enzymes. 3βHSDH is needed for the production of progesterone while

17βHSDH is needed for estradiol synthesis. Our result showed dose dependent and time dependent effect where cadmium treated animals were affected the most while combined animals showed intermediate results in both reproductive tissues. The extent of inhibition of 3βHSDH in proestrous stage is more than estrous stage while 17βHSDH showed greater inhibition in estrous stage. This differential effect could be related to difference in expression of the enzymes, responsible for synthesis of sex steroids in different stges of estrous cycle. Apart from the inhibitory effect on steroidogenic enzymes, both hormones estrogen and progesterone demonstrated a decrease, which is partly correlated with the extent of inhibition. However, decreased levels of gonadal hormones could be due to effect of these metals on hypothalamus (GnRH release) (Sierra and Tiffany-Castiglioni, 1992; Vagra and Paksy, 1991) and pituitary (FSH and LH release) supported by earlier reports where single metal exposure at higher dose resulted in decreased hormone levels (Zeng et al., 2003; Sokol et al., 2002).

The inhibition of the steroid dehydrogenases in both the tissues could be attributed to the direct interaction of metals with the enzymes or competition with divalent ions like zinc and calcium (Flora *et al.*1982; Waakles & Poirier 1985; Paksy *et al.* 1996). Both the key enzymes belong to the class of short chain alcohol dehydrogenases, which contain the Tyr-X-X-X-Lys at its active site (Persson et al., 1991). The metal ions lead and cadmium can interact with these amino acid residues or get bound to the –SH groups of cysteine residue present at the NAD binding domain (Persson et al., 1991) and alter the structure so that the substrate binding and function gets significantly affected. The intermediate effect observed in combined treated group could be correlated either to the amount of metals accumulated (which is intermediate in combined treated group) or competition of two metals for a single binding site.

In case of time dependent study, lead and combined treatment causes time dependent inhibition of steroidogenic enzymes while cadmium treated animals does not show a similar a decrease in inhibition as the time of exposure increases especially from 30 to 45 days. This suggests that there could be saturation of metals at the active site of the enzyme.

In case of uterus too, steroidogenic enzymes exhibited a time dependent inhibition except for cadmium group. Decreased expression of 30-34 and 60-66 kDa proteins has been observed from SDS PAGE, where lead treated animals again showed pronounced effect. It is well documented in literature that StAR protein is of 34 kDa protein that plays an important role in mobilization of cholesterol from cytosol to mitochondria and thus plays an important role in steroidogenesis and 66 kDa protein is a estrogen receptor protein which plays an important role in estrogen function. Thus, it could be speculated that metal treatment caused decreased expression of both the important proteins and thus can modulate uterine function. However, it cannot be stated conclusively since western blot analysis could not be performed. It is also known that uterus expresses Metallothionein, a low weight molecular protein that binds to cadmium (Ioachim et al., 2000; Nishimura et al., 1989). It can be suggested that cadmium administration causes an induction of metallothionein, that binds to all available cadmium and do not allow the excess of metal to demonstrate its effect on steroidogenic enzymes and uterine protein profile with increased time of exposure.

Structural changes caused by accumulation of metals, inhibition of steroid synthesizing enzymes leading to a decrease in hormone profile observed in this study is interesting to note. This study also indicates that metal toxicant exposure at this level, even though is neither showing any additive effect on simultaneous exposure nor sufficient effect to cause clinical signs of toxicity, but still able to manifest biochemical effects and thus have further implications in reproductive health of female population and their progeny. Also, it can be that cadmium being more toxic demonstrate earlier effects but long term

exposure causes an induction of adaptive mechanism and thus manifest less toxic effects than lead and combined exposure mediate an intermediate effect due to competition of two metals.

Summary

Adult female rats were injected intra-peritoneally *(i.p)* with lead acetate and cadmium acetate both separately and in combination in dose dependent manner (0.025, 0.05 and 0.1 mg/kg. body wt/day for 15 days and time dependent manner (15, 30 and 45 days with 0.05 mg/ kg. body wt/ day. Dose dependent accumulation of toxicants, altered structure of ovary and uterus along with a significant inhibition of key enzymes (3β hydroxy steroid dehydrogenase activities and 17 β hydroxy steroid dehydrogenase) activities and decreased levels of steroid hormones were observed. The cadmium treated group showed significant decrease in enzyme activities in dose dependent manner (from 0.025 to 0.05 mg/kg body wt.), but with no change in activity from 0.05 to 0.1 mg/ kg. b. wt. Animals receiving combined treatment showed intermediate results and lead treated group showed minimum change compared to control. This effect was observed in both stages of estrus cycle. From the dose dependent study, 0.05 dose was chosen as optimum dose. Serum and ovarian gonadal steroids were decreased after metal exposure. The enzyme activities of both ovary and uterus decreased as the time of metal treatment increased. Protein profile of uterus showed a change in 30 day and 45 day treated animals. There was a time and dose dependent decrease in serum ALP with slight elevation in SGPT in all metal treated groups. No significant change was observed in hemoglobin and serum creatinine levels. There was a progressive accumulation of metal as dose and time of metal treatment increased. In all treatments, combined treated group showed intermediate results suggesting competition between the two metals.. Simultaneous exposure of metal toxicants at this level neither showed any additive effect nor caused clinical signs of toxicity but still able to manifest biochemical effects and thus affects the ovarian function of treated animals.