INTERNATIONAL JOURNAL OF APPLIED BIOLOGY AND PHARMACEUTICAL TECHNOLOGY

www.ijabpt.com Volume-5, Issue-4, Oct-Dec-2014 Coden : IJABPT Copyrights@2014

ISSN : 0976-4550

Received: 28th July-2014 *Revised: 11th Aug-2014* *Accepted: 11th Oct-2014*

Research article

ANTIENZYMETIC ACTION OF LEAD ACETATE AND ITS POSSIBLE REVERSAL BY ANTIOXIDANT IN TESTICULAR TISSUE OF SWISS ALBINO MICE DURING PUBERTAL PHASE OF LIFE.

Durgesh Nandini Sharma

Endocrinology and Physiology Unit, School of Studies in Zoology and Biotechnology,
Vikram University, Ujjain (M.P.) 456010 India
Corresponding author: dr.durgeshnandini@rediffmail.com

ABSTRACT: Lead intoxication has been associated with male reproductive toxicity in experimental animals and lead may have the potential to produce adverse effects on enzymatic activity in testicular tissue of Swiss albino mice. The present study was undertaken to investigate the ability of antioxidant (Vitamin E) to protect against lead acetated (LA) induced testicular enzymatic toxicity in male albino mice during pubertal phase of life. The weight of testis, caput epididymidis, cauda epididymidis, vas deferens and testicular enzymatic activity (Glutathione peroxidase (GSH-Px), Succenate dehydrogenase (SDH), 65–3β hydroxysteroid dehydrogenase (65–3β–HSD) and 17β–hydroxysteroid dehydrogenase (17β–HSD) were studied. Administration of LA at a dose of 1.25mg/kg body weight for 45 days lowered the weights of testes, caput epididymidis, cauda epididymidis, vas deferens and decreased the activities GSH-Px, SDH, 65–3β–HSD and 17β–HSD. Coadministration of vitamin E (2 mg/kg BW) to the LA group restored all the parameters cited above to near the control values. Therefore, this study revealed that vitamin E has beneficial effects against LA induced enzymatic toxicity in testicular tissue of mice.
Key words: Albino mice, Enzymes, Lead Acetate, Testes, Vitamin E.

INTRODUCTION

The diverse deleterious health effects upon exposure to heavy metals in the environment are a matter of serious concern and a global issue. Lead is the most abundant toxic metal in the environment (Patra *et al.,* 2011) Lead does not have any detectable beneficial biological role however on the contrary its detrimental effect on physiological, biochemical and behavioral dysfunctions have been documented in animals and humans by several investigators (Ruff *et al.,* 1996) Lead is a male reproductive toxicant (Sallmen, 2001). Toxicity is manifested in male reproductive function by deposition of lead in testes, epididymis, vas deferens, seminal vesicle and seminal ejaculate. Lead has an adverse effect on sperm count, sperm motility and retarded the activity of alive sperm (Chowdhury, 2009). Clinical and animal studies indicate that abnormalities of spermatogenesis result from toxic exposure (Ati Hamadouche *et al.,* 2009). The mechanism behind lead toxicity is the oxidative stress and it develops when there is an imbalance between the generation of reactive oxygen species and the scavenging capacity of antioxidants in the reproductive tract. Reactive oxygen species (ROS) have been shown to have an important role in the normal functioning of a reproductive system and in the pathogenesis of infertility (El-Tohamy, and El-Nattat 2010). Accumulated evidence has revealed that testicular enzymology which is basically characterized by steroidogenesis process, gets disrupted, at least in part, by oxidative stress mechanisms (Biwas and Ghosh, 2004). Studies in male rats have shown that lead intoxication disrupts testicular steroidogenesis by inhibiting the activities of testicular steroidogenic enzymes (Liu *et al.,* 2008). Enzymes are one of the major targets for metalloid action. Measurement of certain patterns of cellular enzymes under different conditions of treatments with various types of toxicants could provide good evidence for the cytotoxicity and hence the impairment of cell function (Lavitschka *et al.,* 2007). Reproductive toxicity is the adverse effects of chemicals on gonadal structure and functions, alterations in fertility and impaired gamete function (Timbrell, 1995).

The treatment of lead poisoning, especially at sub clinical level is equally important. Most of the chelating agents tend to have adverse side effects and the benefits are usually transitory, since, blood lead can be rapidly replaced from the bone store (Mahaffey *et al.,* 2000). Oxidative damage associated with the presence of lead has been illustrated as one possible mechanism involved in lead toxicity (Adoneylo and Oteiza, 1999), which suggests that antioxidant might play a role in the treatment of lead poisoning (Gurer *et al.,* 2001).Animals have protective mechanism in the form of antioxidant nutrients, vitamins and several enzymes. Antioxidant may play an important role in abating some hazardous effects of lead. The body consists of an elaborate antioxidant defense system that depends on dietary intake of antioxidant vitamins and minerals. Chow (1991) reported vitamin E and occupies an important and unique position in the overall antioxidant defense. The antioxidant function of vitamin E is closely related to the status of many dietary components. Antioxidative properties of vitamin E is believed to prevent reproductive disease associated with oxidative stress (Brigelius-Flohe *et al.* 2002). Vitamin E interacts with oxidizing radicals and terminates the chain reaction of lipid peroxidations (Jones *et al.,* 1995). In many studies, vitamin E neutralizes lipid peroxidation and unsaturated membrane lipids because of its oxygen scavenging effect (Kalender *et al.,* 2006) El-Shenawy *et al.,* (2010) showed that vitamin E has a preventive and reducible role against the oxidative stress induced by a toxic substance in the testes. The effect of lead acetate on testicular enzymes and its mechanism of action on the male gonads have not been studied. Therefore, the present study has been undertaken on swiss albino mice to investigate the effects of lead acetate on testicular enzymatic activities and weight of accessory reproductive organs and their protection by antioxidant.

MATERIALS AND METHODS

Animals

Healthy adult male swiss albino mice (*Musmusculus*) weighing 35 to 40 g. were used for the experiment. Animals (80 to 90 days) were maintained under standard laboratory condition and provided them balance diet and water ad-libitum daily.

Treatments

Animals were divided into control, experimental and recovery groups. The control group was given vehicle only. The experimental groups were given lead acetate (1.25 mg/kg) daily for 45 days by gavage (0.2 ml/animal). The recovery groups received lead acetate (1.25 mg/kg) and vitamin E (2 mg/kg) for the same period by the same route. The treatment duration of 45 days was selected as the length of the complete spermatogenic epididymal maturation cycle in mice. The doses selected were based on previous work in our laboratory (Sharma and Bhattacharya, 2014). Animals were sacrificed after their respective treatments and their testis, caput epididymidis, cauda epididymidis and vas deferens weights were recorded with an automatic balance (AND GX-600, Japan).

Measurement of testicular enzymes

Glutathione peroxidase (GSH-Px)

The testicular enzyme activity was assayed by the modified technique of Paglia and Valentine (1976). A known tissue weight was homogenized in the required volume of 0.01% digitonin and centrifuged at 12,000 g for 30 min at 4^0C. This supernatant (0.1 ml) was used in the reaction mixture of 0.8 ml. The reaction was initiated by addition of 0.1 ml H_2O_2. The decrease in absorbance at 340 nm was recorded for 3 min at an interval of 1 min to calculate enzyme activity.

Succenate dehydrogense (SDH)

The activity of SDH in the testis was assayed by the method of Beatty *et al.,* (1966). To the sample tube containing 0.4 ml of tissue homogenate in cold distilled water, 1ml sodium succinate and 1ml 2,4-iodophenyl-3,4-nitophenyl-5-phenyl tetrazolium chloride (INT) was added and incubated at 370C for 15 min. In the blank, INT was replaced by 1 ml distilled water. The reaction was terminated by 0.1 ml of 30% trichloroacetic acid. The resulting formazan was extracted in 7 ml ethyl acetate and colour intensity was measured at 420 nm in a Spectronic 106 colorimeter. The activity was expressed as µg formazan/100 mg fresh tissue weight.

3β–HSD and 17β–HSD

One testis from each animal was used for studying the activities of 65–3β–hydroxysteroid dehydrogenase (65–3β–HSD) and 17β– hydroxysteroid dehydrogenase (17β–HSD). Testicular 65–3β –HSD and 17β–HSD were measured in UV spectrophotometer according to the procedure of Talalay (1962) and Jarabak *et al.,* (1962) and subsequently modified by Biswas *et al.,* (1983, 2001). One unit for both 65–3β – and 17β–HSD was defined as the amount causing a change in absorbance of 0.001/min at 340nm.

Statistics analysis

For all biochemical estimation a minimum of 10 to 12 replicates were used for each parameter and tissue. The data were statistically analyzed using ANOVA followed by Scheffe's test for multiple pairwise comparisons (Gad and Weil 1989). A significant level of $p \leq 0.05$ was accepted.

RESULTS

Organ weights

The weights of testis (P <0.01), caput epididymis (P <0.05) , cauda epididymidis (P <0.05) and vas deferens (P <0.05) were significantly lower in mice treated with LA for 45 days compared to control, while vit.E + LA group did not show any significant difference in organ weights compared to control (Table 1).

Assessment of biochemical changes

To determine the testicular damage caused by LA and the protective effect of vitamin E, the activities of some testicular enzymes (GSH-Px, SDH, 65–3β – HSD and 17β– HSD) were used as biomarkers of the testis. After 45 days of LA administration, several changes of the parameters have been observed to indicate the occurrence of testicular injuries by comparing to control group. In this investigation, a highly significant (P<0.001) decrease in GSH-Px and SDH activities were observed after LA intoxication. LA treatment also resulted in a reduction in 65–3β – HSD and 17β– HSD activities in LA intoxicated mice. These enzymes activities did not differ from control value when vitamin E was co administered with LA (Table 2).

Table 1: Organ weights (mg) control and experimental groups of mice.

Parameters	Control	Treated (LA) for 45 days	Treated (LA+Vit.E) for 45 days
Testes weight	123.5±4.2	87.5±6.2**	119.1±1.0^{NS}
Caput epididymidis	27.8±0.7	19.7±0.5*	28.5±0.1 ^{NS}
Cauda epididymidis	19.5±0.7	10.3±1.3*	17.2±0.6 ^{NS}
Vas deferens	14.0±0.4	7.1±0.2*	12.9±0.6 ^{NS}

All values are expressed + SEM, Significant level, NS= Non significant, * = (P<0.05), **= (P<0.01), compared with control, treated (LA) and control treated (LA+Vit.E).

Table 2: Biochemical parameters of control and experimental groups of mice.

Parameters	Control	Treated (LA) for 45 days	Treated (LA+Vit.E) for 45 days
Glutathione peroxidase (mU/mg/min)	0.036±0.01	0.05±0.001***	0.039±0.004 ^{NS}
Succinate dehydrogenase (µg formazan formed/15 min/100 mg)	465±12.13	233±19.33***	435±12.80 ^{NS}
65–3β – HSD (unit/mg tissue per h)	26.39±0.141	20.09±0.140*	24.35±0.124 ^{NS}
17β– HSD (unit/mg tissue per h)	27.82±0.58	21.62±0.43*	25.98±0.79 ^{NS}

All values are expressed + SEM, Significant level, NS= Non significant, * = (P<0.05), **= (P<0.01), *= (P<0.001), compared with control, treated (LA) and control treated (LA+Vit.E).**

DISCUSSION

Lead is multifactorial and directly interrupts enzyme activation, competitively inhibits trace mineral absorption, binds to sulfhydryl proteins (interrupting structural protein synthesis), alters calcium homeostasis, and lowers the level of available sulfhydryl antioxidant reserves in the body (Sharma *et al.,* 2009). In the present study, we observed that lead acetate administration caused testicular dysfunction by disturbing various biochemical parameters such as GSH-Px, SDH, 65–3β – HSD and 17β– HSD.

The present study demonstrated that the activities of GSH-Px and SDH in the testicular tissue were significantly declined in LA-group comparing with controls. Several studies reported alterations in antioxidant enzyme activities such as SOD, catalase and glutathione peroxidise (GPX) and changes in the concentrations of some non-enzymatic antioxidant molecules, such as glutathione (GSH) in lead exposed animals (McGowan *et al.,* 1986) and workers (Gayathri *et al.,* 2007; Mohammad *et al.,* 2008). These findings suggest a possible involvement of oxidative stress in the pathophysiology of lead toxicity. Testicular steroidogenic enzymes (65– 3β –HSD and 17β–HSD) activities decreased after LA treatment. Our result is in agreement with Biswas and Ghosh (2004) who observed low activity of Δ5–3β–hydroxysteroid dehydrogenase (Δ5–3β–HSD) and 17β– hydroxysteroid dehydrogenase (17β–HSD) in the testicular tissue of rats exposed to lead at dose of 8.0mg /kg i.p. over a period of 14 days. Batra *et al.,* (2001) observed a dose dependent reduction in the activity of two major enzymes in the testis, Alkaline phasphatase and Na-K ATPase, in lead exposed animals which is another probable mechanism of lead induced reproductive toxicity. During this investigation, testis and other accessory sex organ weighs were significantly reduced after LA treatment. Macroscopic changes in accessory sex organs such as diminished weight of testes, seminal vesicles, epididymis, and ventral prostate have been demonstrated in various studies using experimental animals (Ronis *et al.,* 1996). Microscopic changes, histological as well as macroscopic ones, have been induced by increasing lead levels in lead exposed male rats (Adhikari *et al.,* 2001) including changes in the testicular tissues morphology (Bonde *et al.,* 2002), and decreased germ cells layer population (Batra *et al.,* 2001).Moreover testicular size and weight are normally regulated by fluid secretion from Sertoli cells and the production of sperm in the seminiferous tubules (Waites and Gladwell 1982). Reduction of testicular weight less than control values after 45 days of lead administration is in support of degeneration of germ cells and sertoli cells in lead-treated rats. Our studies indicate that lead causes disturbances in metabolism of reproductive organs by alterations of biochemical parameters due to oxidative stress.

Antioxidants provide a defense mechanism through 3 levels of protection- prevention, interception and repair. In a normal situation, the cellular antioxidant mechanisms present in almost all tissues and their secretions are likely to quench those reactive oxygen species (ROS) and protect against oxidative damage (.Jones *et al.,* 1979). In the present study, it was observed that vitamin E is a potent antioxidant or free radical scavenger which reduces the lead toxicity in Swiss albino mice testes. The beneficial effects of vit. E can be attributed to the antioxidant effects of this vitamin; it is scavenger of oxygen-free radicals which are toxic byproducts of many metabolic processes (Yousef 2010). Oda and El-Maddawy (2012) reported that the beneficial effect of vit. E is mostly due to its antioxidant properties.

Vit. E protects critical cellular structures against damage caused by oxygen-free radicals and reactive products of lipid peroxidation (Yousef 2006). Moreover, vit. E is essential in maintaining the physiological integrity of testis, epididymis and accessory glands (Cerolini *et al.,* 2006). Conversely, deficiency of vit. E may lead to detrimental effects on the reproductive organs, such as degenerative spermatogonium, testicular damage and degeneration of the seminiferous tubules (Yousef 2010). Treatment of vit. E with LA caused a significant ($P < 0.05$) increase in activity of testicular enzymes GSH-Px, SDH and also increases in activity of testicular steroidogenis enzymes 65–3β – HSD and 17β– HSD. These results are in agreement with the findings by Mishra and Acharya (2004) who found that supplementation of vitamin E and C (100 mg/kg/body weight) along with lead acetate (10 mg/kg/body weight) prevents the lead induced oxidative damage of germinal cells of male mice. Similarly Chinoy and Sharma (1998) reported amelioration of fluoride toxicity by vitamin E and D in reproductive organs of male mice. Ghosh *et al.,* (2002) reported that vitamin C and E ameliorate oxidative stress related testicular impairment in animal tissue. The present study showed that treatment of Vit. E with LA did not show any significant difference in the weight of testes and other accessory reproductive organs weight and activities of testicular enzymes indicating the protective role of vitamin E as an antioxidant.

CONCLUSION

From the current results, it can be concluded that concurrent administration of vitamin E to LA treated animals ameliorated the induced weight and testicular enzymes damage. This is consistent with a vital role of vitamin E in antioxidant systems that protect against LA damage, possibly by preventing oxidative damage to testes. The present studies suggest therapeutic effects of vitamin E to minimize the testicular enzymatic toxicity of LA exposure.

ACKNOWLEDGEMENT

Author is thankful to School of Studies in Zoology and Biotechnology, Vikram University, Ujjain (M.P.), for providing necessary laboratory facilities during this investigation.

REFERENCES

Adhikari, N., Sinha, N., Narayan, R., Saxena, D.K. (2001). Lead-induced cell death in testes of young rats. J Appl Toxicol (21): 275-277.

Adonoylo, V.N. and Oteiza ,P.L. (1999). Lead intoxication: Antioxidant defenses and oxidative damage in rat brain. Toxicology, 15: 135(2-3): 77-85.

Ait Hamadouche, N., Slimani, M., Merad-Boudia, B. and Zaoui, C. (2009). Reproductive toxicity of lead acetate in adult male rats, American Journal of Scientific Research, 1(3): 38-50.

Batra, N., Nehru, B and Bansal, M.P. (2001). Influence of lead and zinc on rat male reproduction at biochemical and histopathological levels. J. Applied. Toxicol, (21): 507-512.

Beatty, C.H., Basinger, G.M., Dully, C.C., Bocek, M.M. (1966). Comparison of red and white voluntary skeletal muschle of several species of primates. J. Histochem. Cytochem. (14): 590-600.

Biswas, N.M., Ghosh, P.K., Neuhaus, O.W. (1983). Effect of α2u- globulin on serum concentration of gonadotropins and testicular activity in oestrogen24 – treated rats. J Endocrinol. (96): 321 – 327.

Biswas, N.M., Roy, Chaudhuri, G. (2001). Effect of casein diet on gonadotropin releasing hormone antagonist induced changes in adrenal gonadal functions in male rats. Indian J.Exp Biol. (39): 1249 – 1253.

Biwas, N.M. and Ghosh, P. (2004). Effect of lead on male gonodal activity in albino rats. Kathmandu University Medical J. 2 (1): 43-46.

Brigelius – Flohe, R., Kelly, F.J., Salonen, J.T., Neuzil, J., Zingg, J.M., Azzi, A. (2002). The European perspective on vitamin E: current knowledge and future research. Am J Clin Nutr, 76 (4): 703-16.

Cerolini, S., Zaniboni, L., Maldjian A. and Gliozzi, T. (2006). Effect of docosahexaenoic acid and tocopherol enrichment in chicken sperm on semen quality, sperm lipid com- position and susceptibility to peroxidation. Theriogenology, (66): 877– 86.

Chinoy, N.J. and Sharma, A.K. (1998). Amelioration of fluoride toxicity by vitamin E and D in reproductive function of male mice. Fluoride, 203-216.

Chow, C.K. (1991). Vitamin E and oxidative stress. Free Radic Biol Med, 11(2): 215-32.

Chowdhury, A.R. (2009). Recent advances in heavy metals induced effect on male reproductive function- A retrospective, Al Ameen Journal of Medical Sciences, 2(2): 37-42.

El-Tohamy, M.M. and El-Nattat, W.S. (2010). Effect of antioxidant on lead-induced oxidative damage and reproductive dysfunction in male rabbits, Journal of American Science, 6(11): 613-622.

Gad, S.C. and Weil, C.S. (1989). Statistics of toxicology In: Hayes AE, editor, Principals and methods of toxicology. 2nd ed. New York: Raven Press, p. 450.

Gayathri, M., Rao Beena, V., Shetty, Sudha, K. (2007). Evaluation of lead toxicity and antioxidants in battery workers. Biomed. Res., 19(1): 1-4.

Ghosh, D., Das, U.B. and Mishra, M. (2002). Protective role of α-tochopherol succinate (provitamin E) in cyclophasphamide induce testicular gemitogenic steroidogenic disorders. Rad. Res., (36): 1199-1208.

Gurer, H. Ozygunes, H. Saygin, E. Erca, l. N. (2001). Antioxidant effect of taurin against lead – induced oxidative stress. Archieves of Environmental Contamination and Toxicology, 41(4): 397-402.

Jarabak, I., Adams, J.A. et al., (1962). Purification of a 17β - hydroxy steroid dihydrogenase of human placenta and studies on its transhydrogenase function. J Biolchem. (237): 345 – 357.

Jones, D.P., Kagan, V.E., Aust, S.D., Reed, D.J., Omaye, S.T. (1995). Impact of nutrients on cellular lipid peroxidation and antioxidant defense system. Fundam. Appl. Toxicol. (26): 1-7.

Jones, R., Mann, T. and Sherins, R.J. (1979). Peroxidation breakdown of phospholipids in human spermatozoa: Spermicidal effects of fatty acid peroxides and protective action of seminal plasma, Fertility Sterility, (31): 531-537.

Kalender, Y., Uzunhisarcikli, M., Ogutcu, A., Acikgoz, F. and Kalender, S. (2006). Effects of diazinon on pseudocholinesterase activity and haematological indices in rats: the protective role of Vitamin E. Environ. Toxicol. Pharmacol, (22): 46–51.

Lavitschka, R., Oliveira, C., Mascara, D., Farial, P., Bincoletto, C. and Esposito, E. (2007). In vitro cytotoxicity and antioxidant activity of Agaricus subrufescens extracts. African Journal of Biotechnology, 6(9):1144-1150.

Liu, H., Niu, R., Wang, J., He, Y., Wang, J. and China, S. (2008). Changes caused by fluoride and lead in energy metabolic enzyme activities in the reproductive system of male offspring rats, Research Report on Fluoride, 41(3): 184-191.

Mahaffey,K.R., McKinney, J., Reigart, J.R. (2000). Lead and Compounds. In: Environmental toxicants : Human Exposures and their health effects, 2/e. Morton lippmann. John Wiley and Sons Inc., pp. 481-52.1

McGowan, C. and Donaldson, W.E. (1986). Changes in organ nonprotein sulfhydryl and glutathione concentrations during acute and chronic administration of inorganic lead to chicks. Biol. Trace Elem. Res., (10): 37-46.

Mishra, M. and Acharya, U.R. (2004). Protective action of vitamins on the spermatogenesis in lead treated Swiss mice. J. Trace. Elements. Medicin. Bio. 173- 178.

Mohammad, I.K., Mahdi, A.A., Raviraja, A. (2008). Oxidative Stress in Painters Exposed to Low Lead Levels. Arh. Hig. Rada. Toksikol. (59): 161-169

Oda, S.S. and El-Maddawy, Z. (2012). Protective effect of vitamin E and selenium combination on deltamethrin-induced reproductive toxicity in male rats. Experimental and Toxicologic Pathology, (64): 813-819.

Paglia, D.E. and Valentine, W.N. (1976). Studies on quantitative and quantitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med. (70):158-169.

Patra, R.C., Rautray, A.K. and Swarup, D. (2011). Oxidative stress in lead and cadmium toxicity and its amelioration, Veterinary Medicine International, (11): 1-9.

Ronis, M.J., Badger, T.M., Shema, S.J., Roberson, P.K., Shaikh, F. (1996). Reproductive toxicity and growth effects in rats exposed to lead at different periods during development. Toxicol Appl Pharmacol. (36): 361-371.

Ruff, H.A., Markowitz, M.E., Bijur, P.E. and Rosen, J.F. (1996). Relationships among blood lead levels, iron deficiency, and cognitive development in two-year-old children, Environmental Health Perspectives, 104(2): 180-185.

Sallmen M. (2001). Exposure to lead and male fertility. Int J Occup Med Environ Health (14): 219-222.

Sharma, D.N. and Bhattacharya, L. (2014). Vitamin E protects testicular oxidative stress and enzymes toxicity induced by led acetate in albino mice. Universal J.Pharmacy. 03 (02): 67-71.

Sharma, V., Kansal, L., Sharma, A. (2009). Prophylactic efficacy of Coriandrum sativum (Coriander) on testes of lead-induced mice, Biological Trace Elements Research, 136(3): 337-354.

Talalay, P. (1962). Hydroxysteroid dehydrogenase in: Colowick, Kaplan eds. Methods in enzymology. New York: Academic Press, (5): 512 –516.

Timbrell, J.A. (1995). Introduction to Toxicology. 2nd ed., London: Taylor and Francis.

Waites, G.M.H., Gladwell, R.T. (1982). Physiological significance of fluid secretion in the testis and blood testis barrier. Physiol Rev. (62): 624 – 671.

Yousef, M. (2010). Vitamin E modulates reproductive toxicity of pyrethroid lambdacyhalothrin in male rabbits. Food and Chemical Toxicology, (48): 1152-1159.

Yousef, M., Awad, T. and Mohamed, E. (2006). Deltamethrin-induced oxidative damage and biochemical alterations in rat and it attenuation by Vitamin E. Toxicology, (227): 240– 247.