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Studies on the Effect of Ambrex (An Amber based Herbal Formulation) on Apoptotic gene Expression and Energy Producing Mitochondrial Enzymes in Isoproterenol Induced Myocardial Infarction in Rats

Rekha Ravindran[1]*, Sekar Sathiya[2], A.Jamuna Devi[1], Chidambaram Saravana Babu[2] and Johanna Rajkumar[1]

[1]Department of Biotechnology, Rajalakshmi Engineering College, Chennai 602 105, Tamil Nadu, India.
[2]Centre for Toxicology and Developmental Research (CEFT), Sri Ramachandra University, Chennai 600116, Tamil Nadu, India.

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Deficiency in energy substrates or stress causes disruption of the Krebs' cycle, leading to a wide range of metabolic disturbances. In the present study an effort was made to unravel the underlying possible mechanism behind the cardio-protective effect of a succinate based herbal formulation Ambrex. Male Sprague Dawley were pre-treated with Ambrex (40mg/kg b.wt./day, p.o) for a period of 21 days. On 20th Day, isoproterenol hydrochloride at 85mg/kg by wt X 2 doses (24h apart) was injected subcutaneously to induce necrosis.Heart tissue mitochondria were isolated for the estimation of mitochondrial enzymes and gene expression of p53, Bax, Bcl2 and Caspase 3 were measured by RT PCR. The characterization of Ambrex was done by FTIR analysis. Isoproterenol-induced myocardial infarcted rats showed significant ($p < 0.001$) increase in the expression levels of apoptotic genes, p53, Bax, Caspase 3and decrease in the anti-apoptotic gene Bcl2($p < 0.001$).Ambrex pretreatment down regulated the apoptotic gene expression. Moreover, the TCA cycle enzymes which were found decreased in the ISPH induced rats showed an enhanced activity in Ambrex pretreated rats. The FTIR analysis showed a sharp absorption peak at 1600 – 1760 cm^{-1} ,assigned to C=O stretching vibration in carbonyl compounds characterized by the presence of high content of terpenoids and flavanoids. Data from the present study suggest that Ambrex prevents the development of cardio-toxicity by a pathway partially related by its ability to increase expression of anti-apoptotic genes and to decrease apoptosis in cardiac tissues with the consequent modulation of the energy producing mitochondrial enzymes, providing insight on the role of succinic acid and other bioactive constituents in the formulation.

Key words: FTIR, apoptosis, Bcl2, mitochondrial, enzymes, herbal, succinic acid, myocardial infarction.

Acute myocardial infarction (MI) results from lack of oxygen supply to the working myocardium. During MI many cells die due to necrosis and those which are less damaged die by apoptosis, and the search is on for drugs that block the process in the hope that some of the apoptotic cell death can be prevented[1]. A damage to mitochondria, the energy reservoir of the cell, leads to cell death. The evidence demonstrating mitochondrial dysfunction due to the oxidative stress induced during myocardial infarction is overwhelming[2]. Increased free radical production thus compromises the ability to meet the energy demands of the cell by reducing the levels of mitochondrial TCA cycle enzymes. In the 21st

* To whom all correspondence should be addressed.
Tel:.+9444000183
E-mail: rekha.ravendran@rajalakshmi.edu.in

century, there is an ongoing effort to integrate complementary and alternative medications into the practice of conventional medicine, for the treatment of cardiovascular diseases[3] . The natural compounds offer a vast chemical diversity due to which it is gaining a lot of importance.[4]

Ambrex is an herb-amber formulation which is a cocktail of four indigenous herbs *Withania somnifera, Cycas circirnalis, Shorea robusta,* and *Orchis mascula* with amber (a resin from *Pinus succinifera*). Amber is the ancient resin of trees which contains succinic acid[5].Many people believe that an amber bracelet will ease rheumatic pain and amber coral bead supposedly help in cases of thyroid illnesses[6].The Research over the last decade has shown that Ambrex has strong therapeutic potential against a variety of tissue damage related disorders[7]. Preservation of mitochondrial integrity is of utmost importance in the design of cardio-protective therapies. Hence in this study an effort has been made to understand the mechanism of cardio-protection of the herbal formulation.

MATERIALS AND METHODS

Chemicals and Reagents

Ambrex, a standardized herbal medicine procured from Care & Cure Herbs Ltd, India. Isoproterenol was purchased from Sigma Aldrich, USA. Himedia laboratories, India and all other chemicals and reagents used were of analytical grade and were procured from SISCO Research Laboratories Pvt. Ltd., India.

FTIR analysis of the Ambrex formulation

The Perkin Spectrum 1 FT-IR instrument was used by pelletizing the sample with KBr using a hydraulic press resulting in a pellet which is used for obtaining the spectra of entire region of 450-4000 cm^{-1}.

Experimental Animals

Male Sprague Dawley rats (150-200g by wt.) were used for the study. Animals were housed in polypropylene cages (6 animals/group; 3 animals/cage) in a well-ventilated room (air cycles: 15/min; recycle ratio: 70:30) under an ambient temperature of 23±2°C and 40–65% relative humidity, with an artificial photoperiod of 12-h light/dark cycle. They were provided with rodent feed (Provimi Animal Nutrition India Pvt. Ltd.) and purified water *ad libitum*. Experimental animals were acclimatized for a period 7 days to the laboratory conditions prior to experimentation. Guidelines of "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, National Academic Press 1996; NIH publication number #85-23, revised 1996) were strictly followed throughout the study. The study was approved by Institutional Animal Ethical Committee (IAEC),Biomedical Research Unit and laboratory Animal Centre (BRULAC), SaveethaUniversity, Chennai, India (IAEC No.Biotech.REC.002/10).

Experimental groups and design

Rats were randomized into four groups of six in each. Group-I and II received (0.5% CMC; 10ml/ kg by wt. /day, p.o.); Group-III and IV received Ambrex (40mg/kg by wt. /day, p.o). Animals were pretreated with vehicle or Ambrex for a period of 21 days. On Day 20 and 21, Group II and IV were injected subcutaneously with ISPH (85mg/kg by wt.) to induce myocardial necrosis[8].The animals were then euthanized following brief CO_2 exposure[9] and the organs such as heart, liver and kidney were collected for further investigation.

Isolation of Heart Mitochondrial Fractions

The heart tissue was put into ice-cold 50 mmol/L Tris-HCl (pH 7.4) containing 0.25 mol/L sucrose and homogenized which were then centrifuged at 700 r/min for 20 min. The supernatant obtained was again centrifuged at 9 000 r/min for 15 min. The pellets were then washed with 10mM Tris-HCl (pH 7.8) containing 0.25 M sucrose and finally re-suspended in the same buffer and used for the estimation of various biochemical parameters.

Biochemical Assays

10% heart homogenate was prepared using 10% KCl. The homogenate was centrifuged at 1000g for 10min and supernatant was collected. The collected supernatant was again centrifuged for 10min at 1000rpm and this step should be repeated for 2-3 times. The post nuclear supernatant was collected from the final step and was again centrifuged at 3300rpm for 10min for 3 times. The pellet collected from each step was pooled and suspended in 1ml of 10% KCl. This extraction was used for the estimation of mitochondrial enzymes.

Gene expression

Reverse transcriptase (RT) - PCR was performed to determine the level of mRNA expression of Caspase 3, Bax, Bcl2 and p53 using the primers shown in Table 1. Briefly, total RNA was extracted from left ventricle (Heart) using TRIzol Reagent (Sigma, USA). The formed cDNA was loaded in agarose gel, allowed to run the electrophoresis at 80V for 30 min and the gene expression was analysed using the bands formed. 200 nanograms of RNA were used for reverse transcription polymerase chain reaction (RT-PCR) according to the manufacturer's instructions (Genet Bio, Korea). The following sequence was performed for each PCR reaction: 42æ%C for 30s, 94æ%C for 5min (1 cycle); 94 æ%C for 1min, Caspase (56.0), Bax (58.8), Bcl_2 (56.7) and p53 (57.9) for 1min, and 72 æ%C for 1 min (with 35 cycles); and a final extension phase at 74 æ%C for 10 min.

Statistical Analysis

Data were expressed as Mean ± Standard error of the mean (SEM). Mean difference between the groups were analysed by one way ANOVA followed by Turkey's multiple comparison as posthoc test. *p* value d" 0.05 was considered to be significant. GraphPad prism 5.0 (San Diego, USA) was used to perform statistical analysis.

RESULTS

FT-IR Spectra

FTIR spectroscopy was useful for the compound identification and was run under IR region in the range of 400 - 4000 cm^{-1} wherein there was a variation in the peaks in the sample. The results (Figure 1) showed that the sample exhibited 13 peaks at 3414, 2929,2134, 1645,1451,1378, 1318, 1243, 1156,1025,780, 761 and 606 cm^{-1} and sample had compounds like Ethers (dialkyl, diaryl) compounds, phosphine oxide(P=O) and phosphine(PH bend) compounds, Imines, Nitro group(aromatic and aliphatic), sulfonates(S-O) and sulfoxides (S=O),primary alcohols and alkenes. The sharp absorption peak at 1600 – 1760 cm^{-1} were assigned to C=O stretching vibration in carbonyl compounds which may show the presence of high content of terpenoids and flavanoids in the complex mixture of Ambrex .The presence of a narrow and sharp peak at ~2929 cm^{-1} were assigned to C-H stretching vibration.

Effect of Ambrex on mitochondrial enzymes

To investigate the effect of Ambrex administration on mitochondrial function, the level of mitochondrial enzymes were measured in cardiac tissues. Activities of the heart mitochondrial TCA

Table 1. Primers sequence of the p53, Bcl2, Bax and caspase 3

S No	Gene Name	Forward primer	Reverse primer
1	Bax	GAGTGTCTCCGGCGAATTG	TGGTGAGCGAGGCGGTGAC
2	Bcl2	CGGGAGATCGTGATGAAGT	CCACCGAACTCAAAGAAGG
3	Caspase 3	CTGGACTGCGGTATTGAG	GGGTGCGGTAGAGTAAG
4	p53	GATGCCCGTGCTGCCGAGGAG	AGTGAAGGGACTAGCATTGTC

Table 2 . Effect of Ambrex on the activities of the heart mitochondrial isocitrate dehydrogenase, succinate dehydrogenase and ± keto dehydrogenase in ISPH induced myocardial necrosis in rats

Groups	Isocitrate dehydrogenase(mg/g tissue)	± - ketoglutarate dehydrogenase(mcg / ml)	Succinate dehydrogenase(mcg/ml)
I	1.74 ± 0.06	31.03 ± 4.11	21.33± 3.98
IIISPH	1.27 ± 0.04***	23.96 ± 2.46**	10.53± 1.09***
III Ambrex	1.76 ± 0.05	32.68 ± 5.48	23.09± 6.90
IVAmbrex +ISPH	1.56 ± 0.08$^{\#}$	28.34 ± 2.02$^{\#}$	17.67± 3.76$^{\#\#}$

[n=6/group. values are expressed in mean ± SEM. Significance with Tukey's test following one way ANOVA is indicated as ** *p* d 0.01 vs Normal control; $^{\#\#}$ *p* d 0.01 vs ISPH group].

cycle enzymes such as ICDH, SDH and ±-KGDH in normal and ISPH- induced rats are shown in Table 2. The activities of these enzymes declined significantly in ISPH-induced rats when compared with normal control rats. Pretreatment of Ambrex at 40mg/kg by wt significantly ($p<0.01$) restored the mitochondrial enzymes in the heart tissue (Group IV). Ambrex pre-treated mice showed significant increases of isocitrate dehydrogenase activity compared with isoproterenol induced group. The activity of ±-KGDH diminished in the isoproterenol group, whereas treatment with Ambrex showed a significant increase in the activity (Pd"0.01) compared to the ISPH group. The activity of succinate dehydrogenase (SDH) decreased in the ISPH group compared with control group. However, ambrex therapy reversed the altered activity of SDH when compared (Pd"0.001) with ISPH group. There was no significant deviation observed in rats treated with Ambrex alone compared to that of control group.

Effect of Ambrex on apoptotic and anti-apoptotic gene expression

Figure 2 shows the effect of isoproterenol, Ambrex alone, and their combination on the p53, Bax, Bcl2 and Caspase 3 mRNA expression level in cardiac tissues. Isoproterenol resulted in a significant increase in p53 ($p< 0.01$) (Figure 5), Bax ($p< 0.01$) (Figure 3) and Caspase 3 (Figure 4) expression level. In contrast, isoproterenol induced a significant decrease in Bcl2 ($p< 0.01$) mRNA expression in cardiac tissues (Figure 6). 21 days treatment with Ambrex significantly decreased p53 and Bax mRNA expression and increased Bcl2 mRNA expression in cardiac tissues. Interestingly administration of Ambrex to isoproterenol treated rats completely reversed the increase in p53, caspase and Bax mRNA expression and the

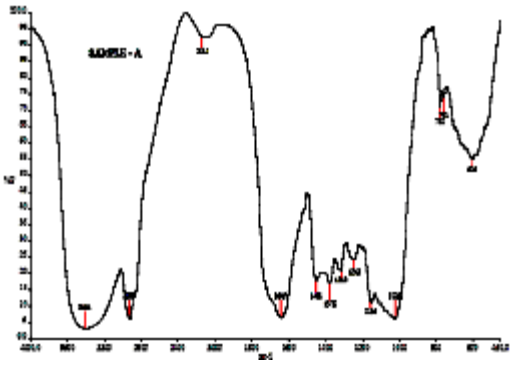
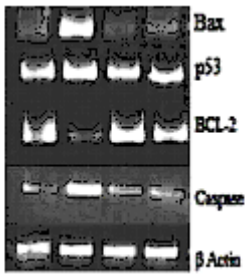


Fig. 1. FT –IR spectra of the Ambrex Formulation



Lane 1 - Normal Control; Lane 2- ISPH treated
Lane 3 - Ambrex (40mg/kg); Lane 4 - Ambrex (40mg/kg) + ISPH

Fig. 2. Effect of Ambrex on apoptotic and anti-apoptotic gene expression

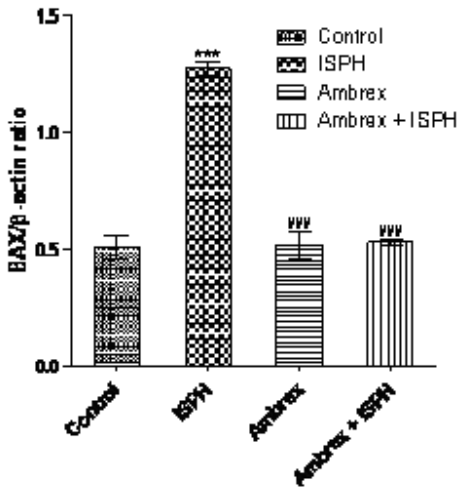


Fig. 3. Effect of Ambrex on Bax gene expression

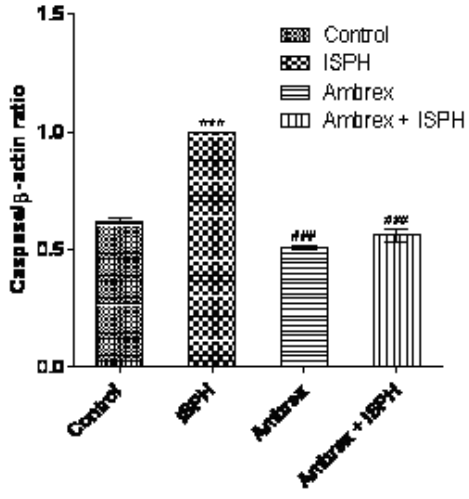


Fig. 4. Effect of Ambrex on caspase gene expression

decrease in Bcl2 mRNA expression, induced by isoproterenol to the control values.

DISCUSSION

During hypoxia and the subsequent generation of lipid peroxides and hydrogen peroxide leads to initiation of chain reactions, can cause mitochondrial damage. The mitochondrial damage due to ISPH induced oxidative stress may affect the activities of TCA cycle enzymes such as isocitrate dehydrogenase (ICDH), Succinate dehydrogenase (SDH) and ±-ketoglutarate dehydrogenase. A decrease in the activities of TCA cycle enzymes was observed in the heart mitochondria of ISPH-induced rats, which are located in the outer membrane of mitochondria and easily affected by excessive production of free radicals by ISPH. Increased free radical production thus compromises the ability to meet the energy demands of the cell by reducing the levels of mitochondrial TCA cycle enzymes. In the present investigation the levels of ICDH, SDH, MDH and ±-KGDH were increased significantly due to Ambrex administration. The dehydrogenase of TCA cycle enzymes could have been affected by the free radicals on isoproterenol induction[10]. Pretreatment with Ambrex to ISO-induced rats significantly increased the activities of these enzymes, which could be due to its ability to prevent free radical formation and free radical scavenging properties of Ambrex.

A deficiency in one or more Krebs's cycle intermediates and an inhibition of normal energy production may cause a wide range of metabolic disturbances and symptoms. Studies have shown that administering specific Krebs' cycle amino acid precursors and intermediates to stimulate energy production significantly reduce symptoms of Chronic Fatigue Syndrome. Succinic acid, like other Krebs' cycle intermediates, is an entry pathway for other metabolites into the cycle and is involved in a variety of important biological actions. Amino acids that are metabolized into succinic acid have been shown to be important in supplying the heart with fuel for myocardium contractions under low oxygen conditions[11]. Supplementing these essential Krebs' cycle acids can enable a partially completed Krebs' cycle to go to completion. They can help correct certain metabolic disorders that result from abnormal mitochondria energy production. Suggesting that Ambrex contains succinic acid had exerted its therapeutic efficacies by regulating metabolites disrupted by MI. The FT-IR study predicted the presence of groups such as OH, CH2, CO, etc. The presence of characteristic functional groups of carboxylic acids, amines, amides, sulfur derivatives, polysaccharides necessary for various medicinal properties of herbal formulation are also present. Each of these compounds play an indispensable role in the complicated system of mixture contributing to the efficacy and potency due to the synergistic effect contributed from a number of constituents present in the herb.

Apoptosis is one of the major processes that lead to the progressive decline of myocardial function responsible for some cardiac pathologies including heart failure, hypertrophy, and myocardial infarction[12]. In heart, apoptosis may take place by

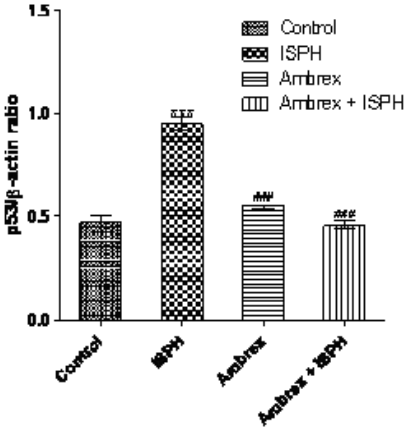


Fig. 5. Effect of Ambrex on p53 gene expression

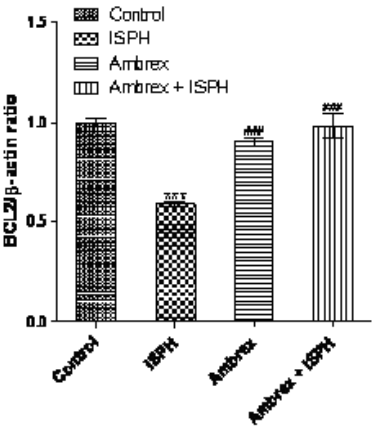


Fig. 6. Effect of Ambrex on Bcl 2 gene expression

death receptor or mitochondrial pathway, where Bcl2 family of proteins play an essential role by the release of cytochrome c, and inhibition of caspase 3 activity[13]. Bcl-2, an apoptosis attenuator, is not expressed in non-infracted myocardial tissue, but it is expressed in cardio-myocytes surrounding infracted areas soon after the onset of infarction[14]. Bax is a member of the Bcl-2 family and when over-expressed, accelerates apoptosis[15] . Cells possess multiple caspases, among which caspase-3 activity is essential to inhibit apoptosis. Under normal conditions, apoptosis is regulated by the balance of a variety of pro-apoptotic (e.g. bax), and anti-apoptotic molecules (e.g. Bcl-2, Bcl-x). Thus, the cardio-protective effect of the succinate based herbal formulation Ambrex has been studied from its treatment regulated apoptic gene expression under biochemical analysis. Ambrex pretreatment regulated apoptotic gene expressions and enhanced the activities of mitochondrial respiratory marker enzymes thereby protecting the myocardium against isoproterenol-induced myocardial infarction. This will open new perspectives for the use of Ambrex in the treatment of myocardial infarction. The use of supplemental Krebs' cycle acids can assist in the management of mitochondrial energy substrates and increase cellular energy production. Such a nutritional approach can be of beneficial to athletes or aged[16], as well as those suffering from metabolic disturbances caused by inherited mitochondrial diseases and other diseases, like Alzheimer's disease and Chronic Fatigue Syndrome[17].

REFERENCES

1. Raff, M. Cell suicide for beginners. *Nature.*, 1998; **396**:119-122.
2. Ansley, D.M., Wang, B. Oxidative stress and myocardial injury in the diabetic heart.*J Pathology.*, 2013; **229**: 232-241.
3. Sharmila, S.T., Rajadurai, M. Preventive effect of bio-aq on cardiac markers, lipids, and membrane bound enzymes in isoproterenol-induced myocardial infarction in rats. *Asian J Pharm Clin Res.*, 2012; **5**: 107-113.
4. Gu, J., Gui, Y., Chen, L., Yuan, G., Lu, H.Z., Xu, X. Use of natural products as chemical library for drug discovery and network pharmacology. *PLoS One.*, 2013; **8**: e62839.
5. Georgiana, I.T., Teodor, E.D., Litescu, S.C., Radu, G.L. LC-MS and FT-IR characterization of amber artifacts. *Cent.Eur.J.Che.*, 2012; **10**:1882-1889.
6. Grimaldi., Still life with flowers. *Natural History.*, 1988; **8**: 86.
7. Jainu, M., Devi, C.S. Effect of ambrex (an amber based formulation) on gastric mucosal damage: role of antioxidant enzymes and lipid profile. *Indian. J. Physiol .Pharmacol .,* 2004; **48**: 343-347.
8. Rona, G., Chappel, C.I., Balazs, T., Gaudry, R. An infarct like myocardial lesion and other true manifestation produced by isoproterenol in the rat. *Arch .Pathol.,* 1959; **67**: 433-455.
9. Zhang, Y., Schlussman, S.D., Rabkin, J., Butelman, E.R., Ho, A., Kreek, M.J. Chronic escalating cocaine exposure, abstinence/ withdrawal, and chronic re-exposure: Effects on striatal dopamine and opioid systems in C57BL/ 6J mice. *Neuropharmacology.*, 2013; **67**: 259-266.
10. Burton, K.P., McCord, J.M., Ghai, G. Myocardial alterations due to free radical generation. *Am J Physiol.*, 1984; **246**: 776-783.
11. Dean, W., English, J. Krebs' Cycle Intermediates. *Nutrition review* 2013.
12. Zhang, Q., Xiang, J., Wang, X., Liu, H., Hu, B., Feng, M., Fu, Q. ²2-adrenoceptor agonist clenbuterol reduces infarct size and myocardial apoptosis after myocardial ischemia/reperfusion in anaesthetized rats. *Br J Pharmacol.*, 2010; **160**: 561-1572.
13. Kirkland, R.A., Franklin, J.L. Bax, reactive oxygen and cytochrome c release in neuronal apoptosis. *Antioxid Redox Signal.*,2003; **5**: 589-596.
14. Virag, J.A., Dries, J.L., Easton, P.R., Friesland, A.M., DeAntonio, J.H., Chintalgattu, V., Cozzi, E., Lehmann, B.D., Ding, J.M., Lust, R.M. Attenuation of myocardial injury in mice with functional deletion of the circadian rhythm gene mPer2. *Am J Physiol Heart Circ Physiol.*,2010 ; **298**: 1088-1095.
15. Lin, J.W., Chen, J.T., Hong, C.Y., Lin, Y.L., Wang, K.T., Yao, C.J., Lai, G.M., Chen, R.M. Honikol traverses the blood brain barriers and induces apoptosis of neuroblastoma cells via an intrinsic bax-mitochondrion cytochrome c-caspase protease pathway. *Neuro Oncol.*,2012; **14**: 302-314.
16. Cadenas, E., Davies, K.J. Mitochondrial free radical generation, oxidative stress and aging. *Free Radic Biol Med.*, 2000; **29**: 222-230.
17. Pieczenik, S.R., Neustadt, J. Mitochondrial dysfunction and molecular pathways of disease. *Exp Mol Pathol.*, 2007; **83**: 84-92.