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Early onset of the maximum protein anabolic effect induced by isoproterenol in chick skeletal and cardiac muscle

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Prolonged (120 days) oral administration of a beta adrenoceptor agonist, isoproterenol hydrochloride (dose = 1.5 mg/kg body weight) resulted in an increase in the live weight of growing chicks (*Gallus domesticus*). Measurement of dry muscle mass and total proteins in muscle homogenates from *M. pectoralis major*, *M. pectoralis minor* suggested a muscle hypertrophy largely responsible for this live weight increase. Further, an increase in organ weight and total tissue proteins supported cardiac hypertrophy in chicks as a result of isoproterenol administration. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed alterations in actin myosin profiles implying a drug induced change in phenotypic expression of myofibrillar component of both skeletal and cardiac muscle. The results suggest that prolonged treatment of chicks produced changes that were not much different from those recorded immediately within a fortnight.

Keywords: beta agonist, isoproterenol hydrochloride, skeletal muscle, cardiac muscle, myosin, actin, growth

Beta adrenoceptor agonists like clenbuterol, isoproterenol and salbutamol demonstrate powerful muscle specific protein anabolic effects (3, 5, 17, 18, 27). These agonists act as sympathomimetic substances which are capable of mimicking normal innervation functions in skeletal muscle. The drugs are reported to stimulate muscle growth by effectively increasing protein accretion in the cells (6). Although the precise mechanism responsible for the increased protein accretion remains debatable, there is a near unanimity over drug-induced increase in protein biosynthesis and a decreased rate of protein degradation (14, 15, 24). These beta adrenoceptor agonists therefore act as potent stimulators of growth and increase protein content and hence tissue mass with a simultaneous decrease in fat contents (4).

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Growth stimulating effects of beta agonists are not only restricted to normal innervated muscles but have been equally documented in different muscle diseases characterized by atrophy of constituent cells. Thus these substances can oppose muscle weaknesses arising in a variety of neuromuscular disorders including denervation atrophy or even reverse dystrophic states (1, 8, 12, 19, 25, 30–32). The beta agonists also promote precocious fusion of myoblasts (26) in addition to their ability to maintain not only total RNA but even increase its translational efficiency in diseased or injured muscle cells (24). These beta adrenoceptor agonists therefore hold great promise in the treatment of muscle wasting disorders and may serve important tool to investigate mechanisms of repair and regeneration in muscle. Treatment of rats with high doses of clenbuterol results in cardiac hypertrophy (7, 22). Chronic use of beta-adrenergic agonist produces deleterious effects on the structure and functions of rat cardiac muscle (7). The purpose of the present study was to investigate relative effects of short-term and long-term administration of isoproterenol in developing chick skeletal and cardiac muscle. More specifically we tested the hypothesis that prolonged treatment of chicks with isoproterenol, a beta agonist, would result in an unabated increase in tissue mass and modify structural components of myofibrillar apparatus mainly myosin and actin and thereby influence functional characteristics accordingly.

Materials and Methods

Day old chicks (*Gallus domesticus*) of white leghorn variety were procured from the Government Poultry Farm at Sundernagar in Himachal Pradesh, India. These were maintained in the animal house of the Department of Biosciences of Himachal Pradesh University under suitable hygienic conditions. These were provided standard poultry feed and water *ad libitum*. The chicks were divided into two groups. Birds of the first group were normal innervated or control chicks. Second group included the normal innervated chicks which received isoproterenol hydrochloride (1.5 mg/kg body weight) orally for varying period of time.

Administration of beta agonist, isoproterenol

Chicks belonging to the second group received isoproterenol hydrochloride (Sigma Aldrich Co. USA) orally in accordance with the following schedule:

(a) Daily administration for the first 15 days.
(b) Administration every alternate day till day 30 of postnatal development.
(c) Administration every 5th day till the 60th day of postnatal development.
(d) Weekly administration till day 120.

Isoproterenol hydrochloride was dissolved in sterilized double distilled water to get a stock solution of 10 mg/ml. Further dilutions were made according to the weight records at the time of oral feeding. A weekly record of body weights was maintained

and changes in the amount of isoproterenol hydrochloride administration formulated accordingly. Each chick was administered 1.5 mg/kg body weight isoproterenol every time.

Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE)

Myofibrillar proteins, viz., myosin and actin were resolved on a 1 mm thick slab gel (12% gel) according to Laemmli (20) as described elsewhere (16). The Coomassie Brilliant Blue R250 stained gels were dried and scanned in a gel scanner (LKB, Broma 2202 Ultrascan Laser Densitometer). Peak area of the respective band was calculated.

Total muscle proteins

Total tissue proteins were extracted from *M. pectoralis major* and *M. pectoralis minor* (supracoracoideus) essentially as per the details described by Haverberg and colleagues (11). In brief, muscle tissue was homogenized in cold distilled water (X 9 vol.) followed by the addition of trichloroacetic acid (TCA) to a final concentration of 10% at 4 °C. Precipitated proteins were separated by centrifugation at 4000 rpm for 30 min at 4 °C. The resultant protein precipitate was washed twice in 10% TCA. Lipids were removed by successive extractions of TCA washed precipitate in (i) ice cold 95% ethanol buffered with 1% potassium acetate, (ii) ethanol : chloroform : 3:1 (v/v) at room temperature, (iii) ethanol : ether 3:1 (v/v; twice) and finally with ether only. Residue was dried to a constant weight in a vacuum drying oven at 45 °C. Lipid free powder was finally weighed to get crude protein content of the muscle tissue. Total protein concentration in respective muscle homogenate was determined according to Lowry and colleagues (21).

Statistical analysis

Data were expressed as mean ± SEM. Statistical significance was determined by the application of analysis of variance (ANOVA) and Student's *t*-test to find out mean differences between groups. Differences were assumed to be significant at $*p<0.05$.

Results

Effects of isoproterenol administration on live body weight and dry muscle mass (Tables Ia and Ib)

Chronic administration of isoproterenol to normal innervated chicks resulted in a significant ($p<0.05$) increase in the live body weight within a fortnight (normal innervated chick = 55.33±0.76 g; normal innervated + isoproterenol treated = 59.33±0.715 g; n = 5 each). With an increase in postnatal development and an

accompanying beta agonist application, increase in live body weight though continuous became relatively inconspicuous (N = 725.40±2.039 g and N + Iso = 740.30±2.906 g; n = 5 each). Maximum increase in live body weight of treated chicks (7.22%) recorded on day 15 declines to as low as 2.05% on day 120.

Table Ia

Changes in total live body weight (in grams) of normal innervated (N) chicks as a result of beta adrenoceptor agonist, isoproterenol hydrochloride (N + Iso = 1.5 mg/kg body weight)

Chicks	Postnatal development (in days)	
	15	120
Normal innervated	55.33 ± 0.760	725.40 ± 2.039
Normal innervated + isoproterenol treated (N+Iso)	59.33 ± 0.715	740.30 ± 2.906

Values are means ± SEM; *p<0.05; n = 5 each

Table Ib

Effects of beta adrenergic agonist, isoproterenol hydrochloride (1.5 mg/kg body weight) administration (N + Iso) on dry muscle mass (mg/g fresh tissue weight) from M. pectoralis major *and* M. pectoralis minor *from normal innervated chicks (N)*

	Postnatal development (in days)	
	15	120
M. pectoralis major (N)	247.50 ± 2.949	288.50 ± 1.170
M. pectoralis major (N +Iso)	262.80 ± 2.122*	302.50 ± 1.330*
M. pectoralis minor (N)	225.50 ± 1.105	268.40 ± 1.376
M. pectoralis minor (N +Iso)	234.70 ± 1.739*	280.20 ± 1.554

Values are means ± SEM; *p<0.05; n = 4 each

Measurement of dry muscle mass in two skeletal muscles, viz., *M. pectoralis major* and *M. pectoralis minor* demonstrated a beta agonist induced increase. Within 15 day post-treatment, dry muscle mass of normal innervated *M. pectoralis major* (N = 247.50±2.949 mg/g fresh tissue weight; n = 4) increased to 262.80±2.122 mg/g fresh tissue weight (n = 4) thus registering a percent increase of 6.18% (p<0.05). On day 120, normal innervated *M. pectoralis major* exhibited a dry muscle mass of 288.50±1.170 mg/g fresh tissue weight; n = 4). The *M. pectoralis major* from treated chicks displayed dry muscle mass of 302.50±1.330 mg/g fresh tissue weight. Increment in dry muscle mass as a result of beta agonist application thus amounted to 4.85% on day 120 (p<0.05). Parallel increments in dry muscle mass of *M. pectoralis minor* as a result of isoproterenol administration (4.07% and 4.39%) were also recorded. Thus, dry muscle mass of normal innervated *M. pectoralis minor* on days 15 and 120

(225.50±1.05 mg/g fresh tissue weight and 268.40±1.376 mg/g fresh tissue weight, respectively; n = 4 each) significantly ($p<0.05$) increased to 234.70±1.739 and 280.20±1.554 mg/g fresh tissue weight, respectively.

Isoproterenol administration induces cardiac hypertrophy in normal innervated chicks (Tables IIa and IIb)

Table IIa

Isoproterenol hydrochloride (1.5 mg/kg body weight) induced changes in weight of heart (in mg) from normal innervated (N) chicks

Heart	Postnatal development (in days) 15	120
Normal innervated	0.505 ± 0.020	3.100 ± 0.147
Normal innervated+ isoproterenol (N + Iso)	0.578 ± 0.010	3.512 ± 0.177*

Values are means ± SEM; *$p<0.05$; n = 5 each

Table IIb

Changes in dry muscle mass (in mg/g fresh tissue weight) of heart from normal innervated (N) chicks as a result of isoproterenol hydrochloride (1.5 mg/kg body weight) administration

Chicks	Postnatal development (in days) 15	120
Normal innervated	209.40 ± 4.319	276.50 ± 1.626
Normal innervated + isoproterenol treated (N+Iso)	220.70 ± 2.266*	293.70 ± 2.816*

Values are means ± SEM; *$p<0.05$; n = 4 each

Measurements of both organ weight and dry muscle mass from control and isoproterenol treated chicks suggested a drug induced cardiac hypertrophy. Organ weight of heart on day 15 (0.505±0.020 g) and day 120 (3.100±0.147 g) changed to 0.578±0.010 g and 3.512±0.177 g, respectively, in chicks which received isoproterenol. Beta agonist application thus resulted in a significant ($p<0.05$; n = 5 each) increase in heart weight. The increase amounted to as high as 14.45% and 13.29% on days 15 and 120 respectively. Simultaneous preparation and measurement of dry muscle mass exhibited an identical pattern of increments. Dry muscle mass of heart on days 15 and 120 in normal innervated chicks was 209.40±4.319 and 276.50±1.626 mg/g fresh tissue weight respectively. Cardiac muscle mass in isoproterenol treated chicks increased to 220.70±2.266 and 293.70±2.816 mg/g fresh tissue weight on days 15 and 120, respectively. The percent increments were 5.39% and 6.22%, respectively ($p<0.05$; n = 4 each).

Effects of isoproterenol on myofibrillar proteins (myosin and actin) (Tables IIIa and IIIb)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis of myofibrillar proteins, viz., myosin and actin revealed a reorganization of their contents in treated chicks. This was established both in skeletal as well as cardiac muscle. Normal innervated *M. pectoralis major* on day 15 demonstrated myosin and actin in the ratio of 62.95±4.39 : 37.05±3.81. This ratio changed to 50.00±4.43 : 50.00±5.08 in treated chicks. The corresponding percent ratio between myosin and actin on day 120 were 57.00±2.83 : 43.00±1.98 and 33.95±3.28 : 66.05±2.93, respectively. Similar changes in myosin and actin proportions were recorded in *M. pectoralis minor* as well. Normal innervated *M. pectoralis minor* exhibited myosin and actin in the percent ratio of 61.09±3.12 : 38.91±2.87 and 56.40±4.07 : 43.60±5.81 on days 15 and 120, respectively. Administration of isoproterenol to these chicks shifted these ratios to 51.75±3.84 : 48.25±4.90 and 39.00±1.89 : 61.00±3.08, respectively. Thus, one of the characteristic effects of beta adrenoceptor agonist in normal innervated chicks included a reorganization of major cytocontractile proteins.

Effect of isoproterenol in reorganization of structural proteins of cytocontractile apparatus were equally well marked in cardiac muscle. Cardiac muscle from normal innervated chick exhibited myosin and actin in the percent ratio of 52.56±4.03 : 47.44±2.14 and 44.06±3.81 : 55.50±4.43 on days 15 and 120, respectively. Administration of beta agonist isoproterenol however, altered these ratios and equilibrium tilted in favour of an increased actin content. Accordingly, the administration of adrenergic agonist isoproterenol hydrochloride changed this ratio to 43.16±1.08 : 56.84±3.32 and 40.44±3.94 : 59.56±3.17 on days 15 and 120, respectively.

Table IIIa

Isoproterenol (Iso) induced alterations in the percent levels of myofibrillar proteins (myosin and actin) in M. pectoralis major *during post-natal development*

Myofibrillar protein	Muscle	Postnatal development (in days) 15	120
Myosin	N	62.95 ± 4.39	57.00 ± 2.83
	N + Iso	50.00 ± 4.43*	33.95 ± 3.28*
Actin	N	37.05 ± 3.81	43.00 ± 1.98
	N + Iso	50.00 ± 5.08*	66.05 ± 2.93*

Values are mean ± SEM; *$p<0.05$; n = 4 each

Table IIIb

Isoproterenol (Iso) induced alterations in the percent levels of myofibrillar proteins (myosin and actin) in M. pectoralis minor *during postnatal development*

Myofibrillar protein	Muscle	Postnatal development (in days) 15	120
Myosin	N	61.09 ± 3.12	56.40 ± 4.07
	N + Iso	51.75 ± 3.84*	39.00 ± 2.39*
Actin	N	38.91 ± 2.87	43.60 ± 5.81
	N + Iso	48.25 ± 4.90*	61.00 ± 3.08*

Values are mean ± SEM; *p<0.05

Table IIIc

Isoproterenol (Iso) induced alterations in the percent levels of myofibrillar proteins (myosin and actin) in cardiac muscle during postnatal development

Myofibrillar protein	Muscle	Postnatal development (in days) 15	120
Myosin	N	52.56 ± 4.03	44.06 ± 3.81
	N + Iso	43.16 ± 1.08*	40.44 ± 3.94*
Actin	N	47.44 ± 2.14	55.50 ± 4.43
	N + Iso	56.84 ± 3.32*	59.56 ± 3.07*

Values are mean ± SEM; *p<0.05

Discussion

We have demonstrated that treatment of normal innervated developing chicks with isoproterenol, a beta adrenoceptor agonist, results in an increase in the body mass primarily as a consequence of skeletal muscle hypertrophy. This is established from increments in the body weights (Table Ia) and dry muscle mass of two muscles, viz., *M. pectoralis major* and *M. pectoralis minor* from treated animals (Table Ib). Hypertrophy was also confirmed from the estimation of total proteins (21) in muscle tissue homogenates. Skeletal muscle tissue comprises the largest tissue mass of the vertebrate body. Manipulations in the weight of muscle tissue therefore certainly influence the body mass. To our knowledge, muscles from birds including chicks have been least tested for growth promoting effects of beta agonists. Hamano et al. (10) reported an increase in weights of breast muscle, gastrocnemius and peroneus longus muscle from broilers following clenbuterol feeding in diet. The present study thus confirms the ability of different beta adrenoceptor agonists in general to promote growth of skeletal muscle in vertebrates as already reported for most commonly employed beta agonist,

clenbuterol in rats and mice (3, 5, 8, 17, 18, 27, 28). Beta adrenoceptor agonists are anabolic agents whose therapeutic potential lies in their ability to increase nitrogen retention and accelerate protein biosynthesis. Use of these anabolic substances in the manipulation of protein metabolism in farm animals including poultry offers a practical way of enhancing meat production. Maximum increase in live weights of treated chicks (7.22%) was recorded on day 15 post-treatment. Level of growth as ascertained from changes in live weights, remained rather subdued or attenuated as the period of treatment/investigation increased. On day 120, increase in the live weight of treated chicks is limited to 2.05% only implying that beta agonists induced increase in live weights is attained within a fortnight and that this increase becomes less conspicuous thereafter. Maximum promotion of growth is attained within a fortnight. Different laboratories (12, 18, 22) have very recently confirmed the effectiveness of beta agonists in enhancing growth, although duration of such an effectiveness remains largely unacceptable on unequivocal terms. Measurement of dry muscle mass employed as an indicator to the retention of N-content or protein levels pointed to a drug induced hypertrophy of both muscles, viz., *M. pectoralis major* and *M. pectoralis minor*, confirming muscle specific anabolic effects of isoproterenol. This implies that beta adrenoceptor agonists in general act as potential growth stimulants and that growth is largely because of protein accumulation. Method employed for measuring dry muscle mass in this investigation also ensured a complete removal of fats/lipids and hence hundred percent residue comprised of proteins. Amongst different beta agonists, clenbuterol has been most commonly studied from the protein anabolic effects point of view. Changes in dry muscle mass of two muscles, viz., *M. pectoralis major* and *M. pectoralis minor* once again suggest that maximum muscle hypertrophy in treated muscles become established within 15 days and that the effects are not compounded thereafter even if the treatment continued till day 120.

One of the important findings of this study is that effects of isoproterenol feeding are not only restricted to skeletal muscle but that cardiac muscle is equally affected. Both an increase in organ weight (Table IIa) and dry muscle mass (Table IIb) support this contention. A pronounced hypertrophy of rat heart was earlier documented (29) as a result of clenbuterol administration. A 28% increase in heart mass has also been reported in rat after year-long clenbuterol treatment (22). Maximum isoproterenol hydrochloride induced hypertrophy of cardiac muscle as apparent from increments in dry muscle mass in this study again appeared as early as within a fortnight. Quantitative variation in hypertrophy became relatively less conspicuous beyond this post-treatment period (13.29% and 6.29% on days 15 and 120, respectively). This is unlike skeletal muscle (12) in which effectiveness of clenbuterol in increasing muscle mass continued for the duration of treatment period. As already discussed, measurement of dry muscle mass of both muscles under investigation here does not support such a belief. Katoch et al. (17) documented maximum growth stimulating effects of clenbuterol in rat gastrocnemius within a week only, though treatment continued for a month. Prolonged treatment with isoproterenol did not modify growth stimulation patterns in the present study.

Measurement of major myofibrillar proteins, viz. myosin and actin in muscles from normal innervated and isoproterenol treated chicks (Tables IIIa and IIIb) suggests a drug induced reorganization or remodeling of the cyto-contractile apparatus. While thick filament protein myosin demonstrates a decline, thin filament protein actin shows a corresponding increase in muscles from treated animals. These changes in thick and thin filament proteins tend to suggest the possibility that structural and functional myofibrillar proteins undergo an extensive reorganization in the presence of beta adrenoceptor agonist isoproterenol. Indirect evidences are already available which suggest that remodeling of myofibrillar proteins following beta agonist application is possible. Evidence for a translational control of beta agonists on myofibrillar proteins synthesis is also available (14). These workers documented a transient rise in actomyosin synthesis in rat gastrocnemius muscle. Myosin heavy and light chains and actin demonstrated an increased incorporation of [^{3}H] phenylalanine. An increased biosynthesis of myofibrillar proteins is thus imminent in treated muscles. Our results point to such an increased myofibrillar proteins synthesis in the presence of isoproterenol. Ji and Orcutt (15) in fowl limb muscle cells *in vitro* reported identical results. It has been concluded that increased muscle protein accretion observed in muscle cells after isoproterenol treatment is probably due to increased protein synthesis. Protein degradation was least affected by beta agonist treatment. Babij and Booth (2) documented a preservation of some α-actin and cytochrome C mRNA in the presence of beta adrenoceptor agonist. Drugs like clenbuterol promote fusion of myoblasts *in vivo* (23) and *in vitro* (26). Possibility of growth stimulation also arises from the fact that beta adrenergic agonists influence the expression of myogenic factors (25) as well. According to Grant and colleagues (9) isoprenaline and ractopamine which have functions similar to clenbuterol increase the rate of proliferation of cultured chicken skeletal muscle cells. Others (9, 13) have reported an elevation in α-actin mRNA in pig muscles treated with ractopamine. Carter et al. (4) documented a reduction in type I MHC in soleus of adult rat with a corresponding emergence of type II mdx MHC isoform as a clenbuterol treatment. All these varied effects of different beta adrenoceptor agonists provide experimental evidence favouring a possibility of beta agonists induced alterations in phenotypic expressions of muscles.

Cardiac hypertrophy as confirmed from increments in organ weight and dry muscle mass is also accompanied by altered myosin actin ratio as a result of isoproterenol treatment (Table IIIc). The two proteins were in the ratio of 53:47 on day 15 in control muscle which changed to 44:56 as development progressed towards day 120. The corresponding figures in the presence of isoproterenol were 43:57 and 40:60, respectively. Peak variations in myofibrillar proteins of heart muscle of treated animals were recorded on day 15 (17.9% decrease in myosin and 19.8% increase in actin). The corresponding figures (decrease in myosin and increase in actin) on day 120 were 8.22% and 7.50%, respectively. Thus, in contrast to skeletal muscle in which effects of isoproterenol treatment on reorganization of myofibrillar proteins continued for the duration of treatment, cardiac muscle typically exhibited maximum changes in myofibrillar proteins profiles within day 15. The effects remained diluted thereafter although drug treatment continued till day 120.

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