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Exposure to Ractopamine Induces Behavioural and Reproductive Alterations in Zebrafish *(Danio rerio)*

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Abstract

The present study was carried out to assess feed additive ractopamine for endocrine disruption in zebrafish. Male zebrafish were exposed to three different doses of ractopamine i.e., 250 ppm, 350 ppm, 450 ppm for 21 days and were observed for behavourial alterations and on mating to unexposed female zebrafish, their embryos were observed for survival and development. It showed that ractopamine was capable of inducing behavioural alterations. There was also effect on male reproduction, which has caused significant reduction in survival and development of embryos over 21 days of exposure. The results demonstrated that ractopamine has the potential to disrupt endocrine system in zebrafish. Therefore, furthermore investigation is necessary in ractopamine production areas for residual content of this drug in water reservoirs for understanding its effects, on the future of animal kind and human kind.

Keywords: Endocrine Disruption, Ractopamine, Zebrafish

1. Introduction

Ractopamine is a member of a class of compound phenylethanolamines, β2 adrenergic receptor agonist used as a feed additive in meat producing animals[1]. It is also called as a 'lean meat agent'[2]. It promotes leanness by stimulating lipolysis and protein synthesis. Due to it steroid like effect and adipolysis there can be possibility of it affecting reproductive physiology[3]. Ractopamine is been used extensively in US and many other countries but its use is been banned in Russian and some Asian countries, as its use is controversial, it is been approved by FDA without analyzing its environmental and public health risks of their use. Ractopamine is said to mimic stress hormone, as administration of it leads to downed animals, muscular tremors and increased aggressive behaviour and cardiovascular dysfunction[4]. But its illicit use still continues, the internet has made even the banned product to be readily accessible for the aim of weight loss or dieting addition[5]. Because of which such drugs are leaching into our waterways contaminating our wildlife and water supplies, and are toxic to our endangered species[4].

Ractopamine can also be potentially introduced into water systems, from the run-off sites receiving waste product from livestock. It is also been reported that ractopamine is a routinely detected drug in highest concentration as compared to the other drugs in the waste

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water near the livestock facility. Besides, ractopamine and other β-agonists are used in the treatment of asthma and pre-term labour in humans. Consequently, hospital effluent is also one of prominent sources of β-agonists in the aquatic environment[6]. It may also leach into the soil and water systems near the ractopamine manufacturing facilities which can affect the aquatic life and also to the human life. As there is very scarce research carried out on the effect of ractopamine on aquatic organisms, our study focuses to assess the effect of ractopamine on the reproduction of aquatic organisms. The present study was carried out according to OECD guidelines 229 for Fish Short Term Reproductive Assay (FSTRA)[22].

Zebrafish species was chosen for the study as it offers several compelling experimental advantages, including transparency of embryo and larva, higher throughput, shorter test period, lower cost, smaller amount of compound required and it is also been approved by U.S. Food and Drug Administration (FDA) and European Medical Agency (EMEA) for toxicity and safety assessments for Investigative New Drug (IND) approval[7]. It also has high genetic homology with humans over 85% as well as important parallels in organogenesis and functional mechanisms[8]. By keeping the above facts in the mind ractopamine was assessed for endocrine disruption in zebrafish by observing for behavioural and reproductive alterations.

2. Material and Methods

2.1 Zebrafish

A total of 200 wild type zebrafish, of 3.5-4 months old were procured from Tata Institute of Fundamental Research, Mumbai, India. They were reared in the facility provided at Department of Pharmacology and Toxicology under optimum temperature 25-31° C. The circadian cycle of 14 hours of light and 10 hours of darkness was strictly maintained. They were housed in breeder tanks and separation tanks depending on the need of the experiment. The water was oxygenated with commercially available aerators. Tanks were cleaned regularly in order to remove debris and maintain fish under hygienic conditions. Feeding schedule of the fishes was as follows (Table 1).

They were acclimatized to the laboratory conditions before commencement of the experiment. Permission from the institutional animal ethics committee was also obtained.

2.2 Experimental Design

Only male zebrafish were exposed to selected three concentrations of ractopamine. For each concentration, two replicates of eight male zebrafish were included. Thus, total of 48 male zebrafish were exposed to ractopamine. Besides, some additional male zebrafish were kept with female zebrafish considering the physiological need for maintaining their reproduction. During mating, they were transferred to breeding tanks free of ractopamine in the ratio of 1 male: 2 female.

Male zebrafish were exposed to different concentrations of the drug through water, as the drug was soluble in the water[9]. Concentrations of ractopamine were selected such that the zebrafish are alive for a period of 21 days[10]. A pilot study was conducted with minimum of 10 adult zebrafish exposed to different concentrations of drug, from which it was concluded that ractopamine is not lethal up to to dose 2000 mg/L, but changes in behaviour like hyperventilation, un-coordinated swimming, loss of equilibrium and altered feeding behaviour were observed.

Table 1. Feeding schedule for fish

Age (In days)	Morning (9.00am)	Afternoon (1.00pm)	Evening (6.00pm)
0-6 days	No feed	No feed	No feed
6-60 days	Larval Diet	Brine shrimp	Larval Diet
60 days onward	Brine shrimp	------	Dry trout Pellet

Based on the behavioural changes, male zebrafish were exposed to three different concentrations of ractopamine viz. 250 ppm, 350 ppm and 450 ppm for a period of 21 days. They were mated with their female partners on every 8th day and the eggs deposited by the females after mating were collected, cleaned and evaluated for spawn, fecundity, mortality and abnormality percent (As per OECD guidelines 229).

2.2.1 Percent Reduction in Spawn Count

The reduction in spawn count at the weekly intervals of 8, 15 and 22 days over the control values for each of the duplicate was calculated. The values thus obtained were utilized for calculating percent reduction in spawn count as follows:

$$\frac{\text{Reduction in spawn count}}{\text{Spawn count (day 01)}} \text{ X } 100 = \text{Percent reduction in spawn count}$$

2.2.2 Fecundity Percent (%)

On gross and microscopic examination the live and dead embryos were distinguished. Live embryos appeared transparent while dead were milky crescent shaped bodies within the egg. Under the inverted microscope (5X), the dead eggs appeared to have a coagulated mass while, the live embryos exhibited the presence of a perfectly circular mass of yolk around which growth was detected during the observation period. The live count for the first day was considered as the initial live count and based on this, the fecundity percent was calculated as follows:

$$\frac{\text{Live count (day 01)}}{\text{Spawn count (day 01)}} \text{ X } 100 = \text{Fecundity \%}$$

2.2.3 Mortality Percent (%)

Once transferred to 96 well plates, the embryos were further evaluated for 72 hpf for any further mortalities. The live and dead embryos observed during this period and the summation of dead embryos or larvae in the 72 hpf gave the total mortality and based on this, the mortality percent was calculated as follows:

$$\frac{\text{Total mortality (0 - 72 hpf)}}{\text{Spawn count (day 01)}} \text{ X } 100 = \text{Mortality \%}$$

2.2.4 Abnormality Percent (%)

During the period of 72 hpf, observations were carried out for abnormalities in the growth and development of the embryo such as delayed hatching of larvae, abnormal yolk formation, heart oedema, body bent. All abnormalities observed were enumerated and recorded as the abnormal count and abnormality percent was calculated as follows:

$$\frac{\text{Total abnormality (0 – 72 hpf)}}{\text{Live count (day 01)}} \text{ X } 100 = \text{Abnormality \%}$$

2.2.5 Statistical Analysis

One way analysis of variance was used for analysis followed by Dunnett's multiple range test. $P \leq 0.05$ was considered as significant difference. Pre-exposure values was considered as control values for considering data of spawn count, fecundity percent, mortality percent and abnormality percent.

3. Results

3.1 Behavioural Test

Group I, II, III treated with ractopamine showed significant changes in the behaviour in the increasing concentration of the drug as compared to control (Table 2).

3.2 Assessment of Reproduction

The potential of ractopamine to affect endocrine system and its effect on male reproductive system was assessed by examination of embryos at 0 to 6, 24, 48 and 72 hpf using the following endpoints:

- Spawn count (total count of eggs)
- Fecundity percent
- Mortality percent
- Abnormal growth count

3.3 Spawn Count

The enumeration of all the eggs collected in the embryo medium post-mating of adult zebrafish gave the total spawn count. Spawn count consisted of enumeration

Table 2. Behavioural changes observed after exposure of male zebrafish to ractopamine for 21 days

	Control	Group I	Group II	Group III
Confused behaviour	0^{a}	2^{b}	3^{c}	4^{d}
Colliding to the walls	0^{a}	3^{b}	5^{c}	7^{d}
Hyper- ventilation	0^{a}	3^{b}	5^{c}	6^{d}
Using less space to swim	0^{a}	4^{b}	6^{c}	8^{d}
Un coordinated swimming	0^{a}	3^{b}	4^{c}	5^{d}
Decreased feeding behaviour	0^{a}	4^{b}	5^{c}	6^{d}
Territorial behaviour	5^{d}	3^{c}	2^{b}	1^{a}

Values are Mean (n=8) One way ANOVA. Means with different alphabets differ significantly (P≤0.05); small alphabets (horizontal comparison). Control: No exposure to RAC. Group I: 1-21 days exposure to 250 ppm of RAC. Group II: 1-21 days exposure to 350 ppm of RAC. Group III: 1-21 days exposure to 450 ppm of RAC. RAC- Ractopamine, SEM- Standard Error of Mean.

Table 3. Total spawn count in zebrafish after exposure of male zebrafish to ractopamine for 21 days

	Group I			Group II			Group III		
	I a	I b	Mean ±S.E.	II a	II b	Mean ±S.E.	III a	III b	Mean ±S.E.
Pre-exposure	1299	1211	1255.00 ± 44.00^{aA}	1247	1274	1260.50 ± 13.50^{aA}	1380	1294	1337.00 ± 43.00^{bA}
Post-exposure Day 8	987	952	969.50 ± 17.50^{aB}	1031	1011	1021.00 ± 10.00^{bB}	1016	915	965.50 ± 50.50^{aB}
Post-exposure Day 15	746	698	722.00 ± 24.00^{aC}	765	771	768.00 ± 3.00^{bC}	693	661	677.00 ± 16.00^{aC}
Post-exposure Day 22	447	380	413.00 ± 33.50^{aD}	421	436	428.50 ± 7.50^{aD}	409	417	413.00 ± 4.00^{aD}

Values are Mean ± SEM (n=8) One way ANOVA. Means with different alphabets differ significantly (P≤0.05); capital alphabets (vertical comparison); small alphabets (horizontal comparison). Group I: 1-21 days exposure to 250 ppm of RAC. Group II: 1-21 days exposure to 350 ppm of RAC. Group III: 1-21 days exposure to 450 ppm of RAC. RAC- Ractopamine, SEM - Standard Error of Mean.

Table 4. Percent reduction in spawn count of exposed male zebrafish over the control values during the 21 day exposure period

	Group I			Group II			Group III		
	I a	**I b**	**Mean ±S.E.**	**II a**	**II b**	**Mean ±S.E.**	**III a**	**III b**	**Mean ±S.E.**
Post- exposure day 8	24.01	21.39	22.70 ±1.31^{bA}	17.32	20.64	18.98 ±1.66^{aA}	26.38	29.28	27.83 ±1.45^{bA}
Post-exposure day 15	42.57	42.36	42.46 ±0.10^{aB}	38.65	39.48	39.06 ±0.41^{aB}	49.78	48.91	49.34 ± 0.43^{bB}
Post-exposure day 22	65.58	68.62	67.10 ±1.52^{aC}	66.24	65.77	66.00 ±0.23^{aC}	70.36	67.77	69.06 ±1.29^{aC}

Values are Mean ± SEM (n=8) One way ANOVA. Means with different alphabets differ significantly (P≤0.05); capital alphabets (vertical comparison); small alphabets (horizontal comparison). Group I: 1-21 days exposure to 250 ppm of RAC. Group II: 1-21 days exposure to 350 ppm of RAC. Group III: 1-21 days exposure to 450 ppm of RAC. RAC- Ractopamine, SEM- Standard Error of Mean.

of both live and dead embryos in the zero to six hours post fertilization (hpf) time period. Percent reduction in spawn count over the exposure period was calculated and the results depicted significant (p≤0.05) reduction at all three recording periods in respect to their pre-exposure spawn count (Table 3&4).

3.4 Fecundity percent (%)

The enumeration of all the live eggs collected in the embryo medium post mating of adult zebrafish gave the initial live count. Live count enumeration was carried out in the pre-exposure as well as the exposure groups

Table 5. Dead count and percent fecundity of embryos collected weekly over the period of 21 days of exposure of male zebrafish to ractopamine

	Group I		Group II		Group III	
	Pre-exposure observations					
	I a	I b	II a	II b	III a	III b
Total spawn count	1299	1211	1247	1274	1380	1294
Dead count	49	47	34	36	42	43
Live count	1250	1164	1213	1238	1338	1251
% fecundity	96.22	96.11	97.27	97.17	96.95	96.67
Mean (±SE)	96.16±0.05^{aA}		97.22±0.05^{aA}		96.81±0.14^{aA}	
Post-exposure observations						
8 days post-exposure						
	I a	I b	II a	II b	III a	III b
Total spawn count	987	952	1031	1011	1016	915

Dead count	290	292	180	187	210	200
Live count	697	660	851	824	806	715
% fecundity	70.61	69.33	82.54	81.50	79.33	78.14
Mean (±SE)	69.97±0.64^{aB}		82.02±0.52^{cB}		78.73±0.59^{bB}	
15 days post-exposure						
	I a	I b	II a	II b	III a	III b
Total spawn count	746	698	765	771	673	661
Dead count	300	288	266	262	330	326
Live count	446	410	499	509	343	335
% fecundity	59.78	58.73	65.22	66.01	50.96	50.68
Mean (±SE)	59.25±0.52^{bC}		65.61±0.39^{cC}		50.82±0.14^{aC}	
22 days post-exposure						
	I a	I b	II a	II b	III a	III b
Total spawn count	447	380	421	436	409	417
Dead count	254	212	267	271	271	279
Live count	193	168	154	165	138	138
% fecundity	43.17	44.21	36.57	37.84	33.74	33.09
Mean (±SE)	43.69±0.52^{cD}		37.20±0.63^{bD}		33.41±0.32^{aD}	

Values are Mean ± SEM (n=8) One way ANOVA. Means with different alphabets differ significantly (P≤0.05); capital alphabets (vertical comparison); small alphabets (horizontal comparison). Group I: 1-21 days exposure to 250 ppm of RAC. Group II: 1-21 days exposure to 350 ppm of RAC. Group III: 1-21 days exposure to 450 ppm of RAC. RAC- Ractopamine, SEM- Standard Error of Mean.

of the concentrations 250 mg/L (Group I), 350 mg/L (Group II) and 450 mg/L (Group III) at weekly intervals in the period of 21 days. Fecundity percent was then calculated from the live count and spawn count obtained. Comparisons of the pre-exposure values to their exposed values indicated significant (p≤0.05) reduction at all three recording periods (Table 5).

3.5 Mortality Percent (%)

The dead embryos observed during 0-72 hpf, were enumerated and recorded as dead count. The summation of dead embryos in the 72 hpf gave the total mortality and based on this, the mortality percent was calculated. Comparison of the pre-exposure values to their exposed

Table 6. Total and percent mortality recorded during post-spawning observation period of embryos collected weekly over 21 days period

	Group I		Group II		Group III	
Control	**I a**	**I b**	**II a**	**II b**	**III a**	**III b**
0-6 hours	10	13	16	08	09	11
24 hours	13	22	20	19	15	15
48 hours	22	23	23	27	19	18
72 hours	23	29	26	28	28	24
Total	68	87	85	82	71	68
% Mortality	5.23	7.18	6.82	6.43	5.14	5.25
Mean (±SE)	6.20 ± 0.97^{aA}		6.62 ± 0.19^{aA}		5.19 ± 0.05^{aA}	
	8 days post-exposure					
	Group I		Group II		Group III	
	I a	I b	II a	II b	III a	III b
0-6 hours	98	94	19	23	29	23
24 hours	81	73	82	77	35	34
48 hours	77	97	104	101	61	63
72 hours	102	87	98	112	91	89
Total	358	351	303	313	216	209
% Mortality	36.27	36.87	29.38	30.95	21.25	22.84
Mean (±SE)	36.57 ± 0.30^{cB}		30.16 ± 0.78^{bB}		22.04 ± 0.79^{aB}	
	15 days post-exposure					
	Group I		Group II		Group III	
	I a	I b	II a	II b	III a	III b
0-6 hours	58	68	38	27	110	107
24 hours	102	94	96	109	118	123
48 hours	136	123	106	107	112	124
72 hours	120	117	102	99	130	98
Total	416	402	342	342	470	452
% Mortality	55.76	57.59	44.70	44.35	69.83	68.38
Mean (±SE)	56.67 ± 0.91^{bC}		44.52 ± 0.17^{aD}		68.10 ± 0.72^{cC}	
	22 days post-exposure					
	Group I		Group II		Group III	
	I a	I b	II a	II b	III a	III b
0-6 hours	22	19	20	19	29	31
24 hours	36	24	32	37	57	59

48 hours	49	47	50	46	64	67
72 hours	53	49	67	69	80	77
Total	160	139	169	171	230	234
% Mortality	35.79	36.57	40.14	39.22	56.23	56.11
Mean (±SE)	36.18 ± 0.39^{aD}		39.68 ± 0.46^{bC}		56.17 ± 0.06^{cD}	

Values are Mean ± SEM (n=8) One way ANOVA. Means with different alphabets differ significantly (P≤0.05); capital alphabets (vertical comparison); small alphabets (horizontal comparison). Group I: 1-21 days exposure to 250 ppm of RAC. Group II: 1-21 days exposure to 350 ppm of RAC. Group III: 1-21 days exposure to 450 ppm of RAC. RAC-Ractopamine, SEM- Standard Error Mean.

values indicated significant (p≤0.05) reduction at all three recording periods (Table 6).

3.6 Abnormality percent (%)

The enumeration of all abnormalities observed in the embryo and early-larval stage maintained in the embryo medium in 96 well plates and observed for 72 hpf under microscope gave the abnormal count. The abnormalities observed in the embryo stage and early larval stage included abnormal yolk formation, heart oedema, granule formation and body bent defect in early larval stage (Figure 1 & 2). The percent abnormal count was calculated from the initial live count and abnormal count readings. Comparison of abnormal embryo count within

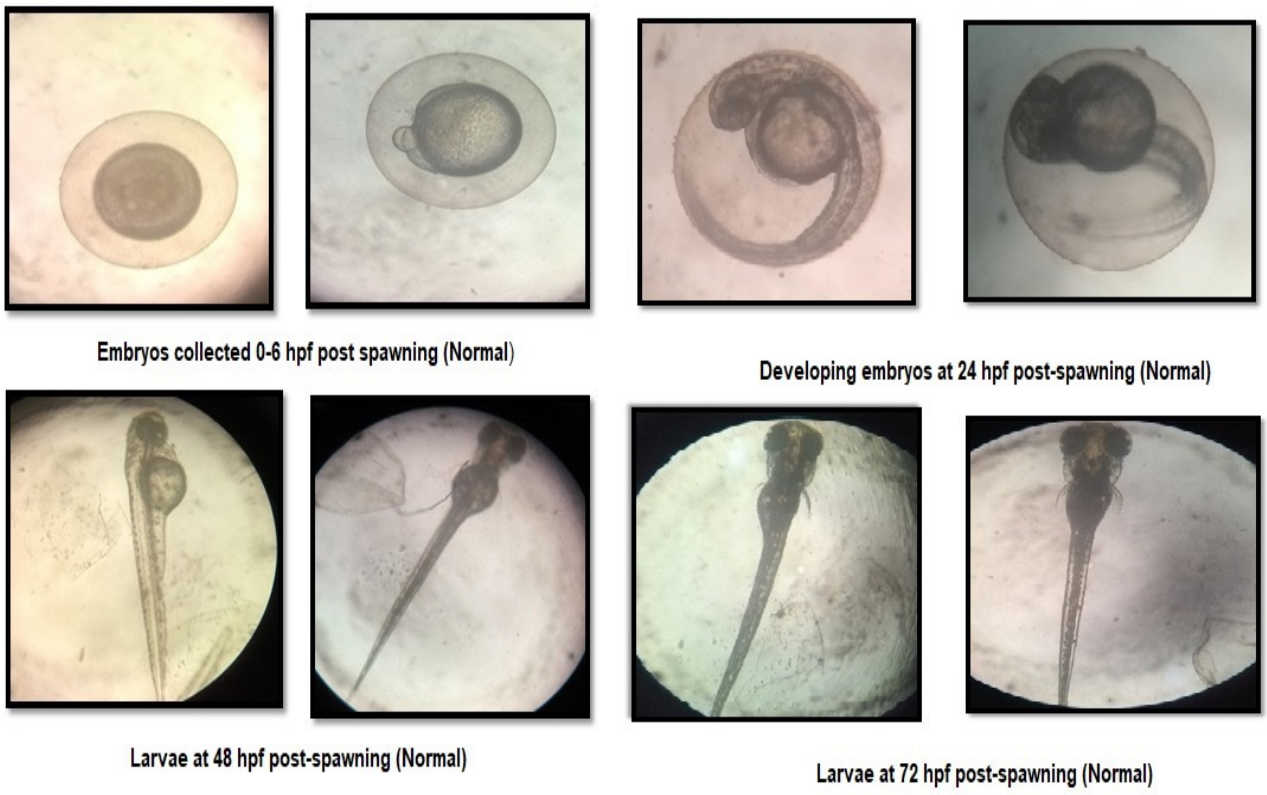


Figure 1. Normal development of embryo.

Table 7. Total and percent embryo defects recorded during post-spawning observation of embryos collected weekly from ractopamine exposed male zebrafish

	Pre-exposure					
	Group I		**Group II**		**Group III**	
	I a	I b	II a	II b	III a	III
Abnormal count	39	38	43	47	51	50
Percent abnormality	3.12	3.26	3.54	3.79	3.81	3.99
Mean (±S.E.)	3.19±0.07^{aA}		3.66±0.12^{aA}		3.90±0.09^{aA}	
	8 days post-exposure					
	Group I		Group II		Group III	
	I a	I b	II a	II b	III a	III
Abnormal count	165	155	170	161	210	195
Percent abnormality	23.67	23.48	19.97	19.53	26.05	27.27
Mean (±S.E.)	23.57±0.09^{bB}		19.75±0.22^{aB}		26.66±0.61^{cB}	
	15 days post-exposure					
	Group I		Group II		Group III	
	I a	I b	II a	II b	III a	III
Abnormal count	240	214	188	214	196	189
Percent abnormality	53.81	52.19	37.67	42.04	57.14	56.41
Mean (±S.E.)	53.00±0.81^{bC}		39.85±2.18^{aC}		56.77±0.36^{cC}	
	22 days post-exposure					
	Group I		Group II		Group III	
	I a	I b	II a	II b	III a	III
Abnormal count	122	108	72	76	104	107
Percent abnormality	63.21	64.28	46.75	46.06	75.36	77.53
Mean (±S.E.)	63.74±0.53^{bD}		46.40±0.34^{aD}		76.44±1.08^{cD}	

Values are Mean ± SEM (n=8) One way ANOVA . Means with different alphabets differ significantly (P≤0.05); capital alphabets (vertical comparison); small alphabets (horizontal comparison). Group I: 1-21 days exposure to 250 ppm of RAC. Group II: 1-21 days exposure to 350 ppm of RAC. Group III: 1-21 days exposure to 450 ppm of RAC. RAC- Ractopamine, SEM- Standard Error Mean.

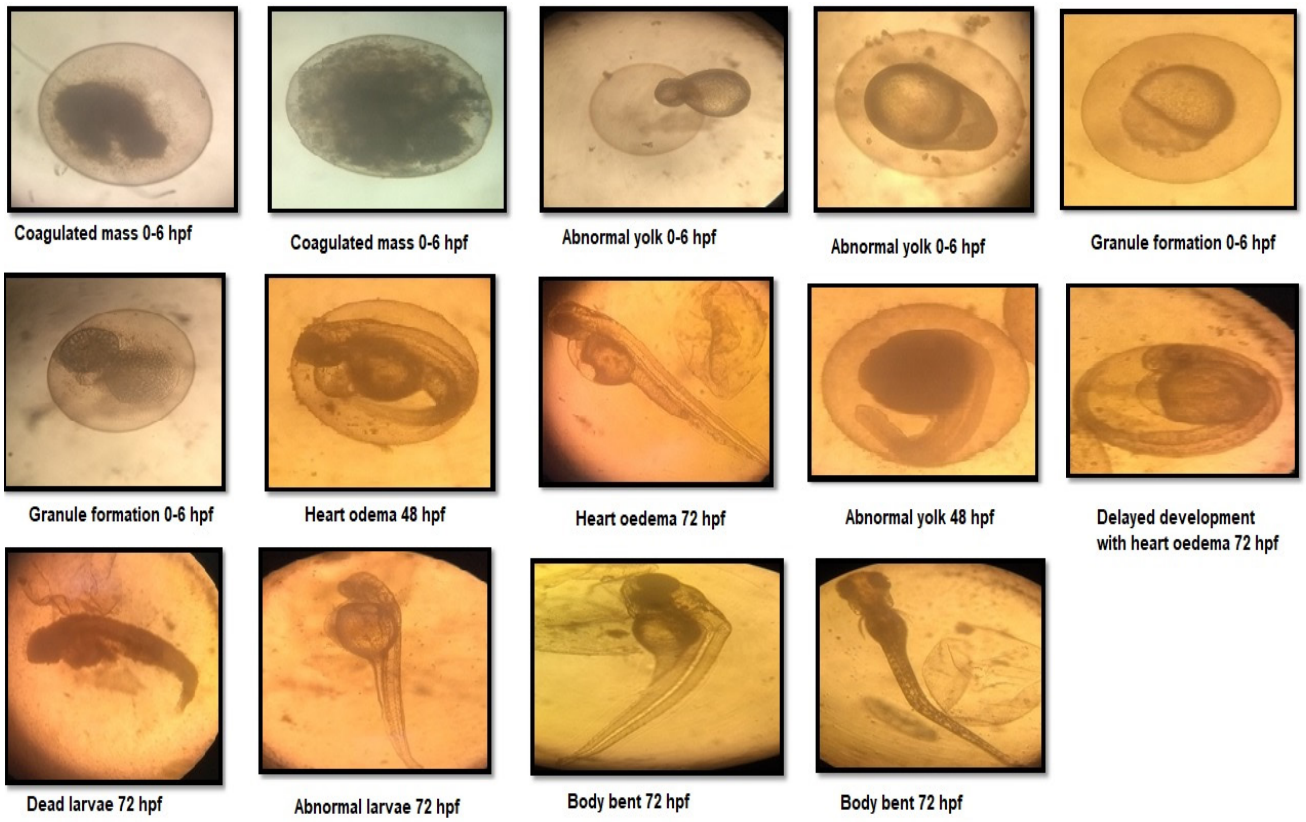


Figure 2. Abnormal development of embryos after exposure to ractopamine.

respective groups to the respective pre-exposure values indicated significant ($p \leq 0.05$ and 0.001) difference at all three recording periods (Table 7).

4. Discussion

Exposure to β-adrenergic agonist clenbutarol, salbutamol and prenaterol on rats suppressed avoidance responding in rats in dose dependent manner and also reduced feed intake, suppressed locomotor activity, inter-trial response and increased avoidance latency[11]. On administration of 1mg/Kg ractopamine to greyhounds it was observed that it caused lethargy, vomiting and abnormal heart rate after two hours of administration[12]. Exposure of ractopamine to nematode *Caenorhabditits elegans* caused decrease in locomotion behaviour[5]. On exposure of ractopamine to zebrafish at the dose rates of 0.1, 0.2, 0.85, 8.5 or 85 µg/L in water it was observed that there was decreased locomotion behaviour and exploratory activities at 8.5 µg/L, and increased exploratory activities at 0.85 µg/L[13]. All the different findings of β-agonistic activity in laboratory animals corroborate the present findings of behaviour on zebrafish.

In the present study, exposure to different concentrations of ractopamine caused significant reduction in the spawn count as compared to the pre-exposure count, which indicates that exposure to ractopamine had affected male reproduction in zebrafish. According to physiological behaviour of zebrafish with regards to its reproduction and spawning, olfactory cues play a determining role. Upon ovulation, females release pheromones that in turn prompt male mating behaviour that immediately precedes and elicits oviposition and spawning[14]. Thus, the reduction in number of eggs spawned by the female might have occurred due to reduction in steroids released by the exposed male zebrafish. It is been reported that exposure to ractopamine to nematode *Caenorhabditits elegans* caused decrease in brood size at both acute and prolonged exposure[5]. Administration of paylean (a commercial product of ractopamine) in pigs

affected services pre conception, number of piglets born alive, weaning of the piglets[15].

In the present study, it was observed that there was significant reduction in fecundity in all the exposed groups at all three recording periods as compared to its pre-exposure values. Fertilization success is probably one of the earliest estimates of sperm quality. The reduction in fecundity indicates that fertilization has reduced with increased period of exposure. Exposure of nematodes *Caenorhabditits elegans* to ractopamine had caused reduction in life span[5].

Significant increase in the mortality count at all the three recording periods in comparison to pre-exposure values, indicated the effect of sperm released by exposed male zebrafish on the livability of the embryo. Administration of β-agonist drug zilpeterol hydrochloride, caused decrease in sperm motility in lambs from this it was concluded that it affects reproductive performance in rams[16]. Studied[2] effect of ractopamine on the 2-cell mouse embryos development *in-vitro*, they observed that ractopamine induced mouse embryos death, stunted development and eventually degraded 2- cell mouse embryos[2]. Found[17] that zilpeterol hydrochloride caused down-regulation of testicular androgen receptors in broiler chickens. Found[18] that β-adrenergic agonist caused alteration in testicular blood flow and also change in testicular temperature which might have affected sperm motility. Found[19] lighter testicles when they include β-adrenergic agonist in veal calves.

In present study, abnormal embryo count within respective exposed groups to the respective pre-exposure values indicated significant increase ($p \leq 0.05$) at all three recording periods. Stunted development, oedema of heart, granule formation and degeneration of yolk, yolk deformities, delayed hatching of embryos were the abnormalities recorded 72 hpf. Investigated[2] the effect of ractopamine on 2 cell mouse embryos development, *in- vitro* at the dose 0.5 µM, 5 µM, 50 µM and reported lower developmental rate, greater number of granules, fragments and degeneration compared to control group[2]. Examined[20] the effects of terbutaline, a β-adrenoceptor agonist on developmental toxicity and observed that it causes apoptosis and altered cell differentiation. Study Observed[21] effect of prolonged administration of β-agonist ritodrine to the mother and noted significant reduction in fetal body weight and skeletal muscle weight.

In conclusion, the present study suggests that ractopamine exposure at 250 ppm, 350 ppm, 450 ppm in water to zebrafish, significantly affects behaviour and male reproductive system in zebrafish.

The present study is an introduction to the importance of Environment Risk Assessment (ERA) in relation with the entry of feed additives like β-agonist into the environment and their effects on the endocrine system of non-target organisms including humans. It was observed that exposure to these concentrations can be detrimental to the development of the off springs and this is a warning for what can be expected in the years to come if appropriate measures are not undertaken to check the entry of chemicals and their accumulations in the environment. In future, studies need to be carried out in ractopamine manufacturing areas for its concentration in soil and water and its effect on environment and on animals and humans around. Also, present study can be done with addition of histopathology, proteomic analysis and hormonal assays of zebrafish.

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