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SHORT COMMUNICATION

Immune effector activities and clinical biochemistry of normal pangas catfish *Pangasius pangasius* (Hamilton, 1822)

Harresh Adikesavalu . Farhana Hoque . Thangapalam Jawahar Abraham

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Abstract The intensive production of catfish is adversely affected by infectious diseases. This study determined the immune effector activities and clinical biochemistry of healthy pangas catfish *Pangasius pangasius* to ascertain the normal ranges for select non-specific and specific immune parameters as well as serum biomarkers. The ranges for immune effector activities like serum lysozyme (192.00–387.78 U/ml), ceruloplasmin (0.22–0.37 optical density (OD) at 540 nm), anti-protease (3.17–10.22 inhibition %), myeloperoxidase (0.12–0.19 OD at 450 nm), respiratory oxidative burst (0.21–0.56 OD at 540 nm) activities, in-vitro nitric oxide production (130.00–510.00 µM) and in-vitro lymphocyte proliferation upon mitogen (Con-A) stimulation (0.92–3.81 OD at 540 nm) were documented. The normal values for serum biomarkers like cortisol (30.15-41.94 µg/dl), glucose (140.33-166.02 mg/ml), alanine aminotransferase (9.37-13.20 IU/L), aspartate aminotransferase (112.60-143.20 IU/L), lactate dehydrogenase (380.63-462.80 IU/L), creatinine (0.17-0.26 mg/ml), C-reactive protein (1.81-2.45 mg/L), insulin-like growth factor-1 (8.30-10.64 ng/ml) and insulin-like growth factor binding protein-1 (3.68-4.99 ng/ml) were also established in *P. pangasius*. Variations in the immune effector activities among the different batches of catfish were observed. These results indicated that the immune effector activities and serum biomarkers levels of *P. pangasius* are different from other catfish species. The observed baseline values of pangas catfish immune effector activities and serum biomarkers could be used as indicators, which would help interpret the clinical and immune responses during the infections and for developing immunoprophylactic measures.

Keywords Catfish . Physiological status . Serum biomarkers . Indicators

Introduction

Fish dwell in a highly dynamic environment, and their physiology is constantly adapting to potential changes in water quality, environmental stressors and pathogens. The fish innate immunity includes both humoral and cell-mediated protective mechanisms, which are the first line of defense, against invading pathogens (Ellis 2001). The non-specific humoral immune defense factors including the acute phase proteins (APPs), found in the serum or plasma that elicit a response to any external stimuli like pathogen infection or tissue injury (Kodama et al. 2004; Swain et al. 2006). The lysozyme is secreted during the non-specific oxygen-independent response by granulocytes, as the first line of defense in fish. Ceruloplasmin can convert molecular oxygen to water by oxidizing ferrous to ferric, which later binds to transferrin for removal (Swain et al. 2006). The antiproteases are enzyme inhibitors that primarily target the internal protein and polypeptide structure of pathogens. Their primary function is to maintain blood homeostasis and to regulate other mechanisms like complement and coagulation during infection (Ellis 2001; Swain et al. 2006). The respiratory oxidative burst (ROB) activity, myeloperoxidase and nitric oxide (NO) production are the non-specific, cell-mediated defense factors found primarily in the phagocytic cells (Sahoo et al. 2005; 2008; Swain et al. 2006). The lymphocyte proliferation is important, as many pathogens evade the non-specific defense factors. The clonal proliferation of fish lymphocytes stimulated by mitogen and also by bacterial antigens has been demonstrated (Adikesavalu et al. 2016). These immune effector activities, however, vary among the teleosts (Grinde et al. 1988; Sahoo et al. 2005; 2008; Swain et al. 2006).

The serum biomarkers have also been effectively employed in monitoring the stress responses, health and

Thangapalam Jawahar Abraham(✉), Harresh Adikesavalu, Farhana Hoque
Department of Aquatic Animal Health, Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences, Chakgaria, Kolkata-700094, West Bengal, India
e-mail:abrahamtj1@gmail.com

physiological status of fish (Refaey and Li 2018; Julinta et al. 2019). Currently, intensive production of catfish is adversely affected by infectious diseases. There has been an increasing interest on the modulation of the immune status of catfish as a prophylactic measure against disease (Welker et al. 2007; Sirimanapong et al. 2014, 2015; Adikesavalu et al. 2016; Sánchez-Martínez et al. 2017). As catfish aquaculture practices continue to expand and intensify, non-lethal and inexpensive diagnostic tools to monitor the fish welfare and health status are necessary. This study aims to ascertain the normal ranges for select non-specific and specific immune parameters as well as serum biomarkers of commercially important pangas catfish *Pangasius pangasius*.

Materials and methods

Five different batches of the experimental pangas catfish, *Pangasius pangasius* (60-190 g) were brought from Kantipota (Lat. 22°27'49" N; Long. 88°24'41" E), South 24 Parganas district and Naihati (Lat. 22°54'10" N and Long. 88°25'01'' E), North 24 Parganas district, West Bengal, India. On receipt, the catfish were disinfected with 5 ppm potassium permanganate solution for 15 min. The active catfish were stocked in circular fibreglass-reinforced plastic (FRP) tanks at 20-50 fish/tank containing 300 L of bore-well water depending on size and acclimatized for three weeks with continuous aeration. The fish were fed twice daily at 3% of their body weight with a balanced commercial pellet feed containing 30% crude proteins, 2% fat and 8% fiber (CP9931, CP Pvt. Ltd., India). The wastes and faecal matter were siphoned out, and 50% water exchange was done once in three days. Periodically, two fish from each batch were randomly screened for the gross and clinical signs to assess the health status. Besides, the fish were euthanized using clove oil (100 µL/L), dissected aseptically and inocula from the kidneys were streaked onto specific bacteriological media to monitor the presence of any disease-causing pathogens.

Healthy *P. pangasius* from the acclimatized stocks were picked randomly, for blood collection and head kidney isolation between March and November. The water temperature was in the range of 22-32 °C during the study period. Six to ten pangas catfish from each batch were anesthetized with clove oil (50 µl/L water) and bled by caudal vein puncture using 2 ml sterile plastic syringe. An aliquot of blood was heparinized using 2.7% EDTA and processed within an hour. The processing of blood and collection and storage of serum was as per Adikesavalu et al. (2016). After the collection of blood, the fish were euthanized by increasing the dose of clove oil to 100-150 µl/L water. The head kidney (HK) was collected following Adikesavalu et al. (2016).

The serum lysozyme level was quantified by the turbidometric method modified to a microtitre plate (Sahoo et al. 2008; Devi et al. 2012). The serum myeloperoxidase, ceruloplasmin and anti-protease activities were determined as per Sahoo et al. (2005; 2008). The determination of the ROB activity of the neutrophils and NO production by macrophages was as described in Mohanty and Sahoo (2010) and Devi et al. (2012), respectively. The proliferative response of the HK leucocytes, as specific immune response, was performed by a tetrazolium based colourimetric assay (Adikesavalu et al. 2016).

The serum glucose, alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatinine and C-reactive protein (CRP) were determined following the test kits (DiaSys Diagnostics systems, GmbH, Germany) in a Photometer (Model: 5010 v5+, Robert Riele KG, Berlin) as described in Julinta et al. (2019). The serum cortisol level was determined by using the cortisol test ELISA kit (AccuBind Elisa Microwells, Monobind Inc., Lake Forest, USA). The serum insulin-like growth factor-1 (IGF-1) and insulin-like growth factor binding protein-1 (IGFBP-1) were determined by ELISA based IGF-1 and IGFBP-1 kits (MyBioSource, San Diego, CA), respectively (Adikesavalu et al. 2016). All the test kits were used as per the manufacturer's protocol. The data in mean and standard deviation (SD) were derived from the MS Excel package.

Results

The levels of serum lysozyme (192.00–387.78 U/ml), ceruloplasmin (0.22–0.37 optical density (OD) at 540 nm), anti-protease (3.17–10.22 inhibition %), myeloperoxidase (0.12–0.19 OD at 450 nm), respiratory oxidative burst (0.21–0.56 OD at 540 nm) activities, in-vitro nitric oxide production (130.00–510.00 µM), and in-vitro lymphocyte proliferation upon mitogen (Con-A) stimulation (0.92–3.81 OD at 540 nm) as immune effector activities of *P. pangasius* are documented in Table 1.

The levels of serum biomarkers like cortisol (30.15-41.94 µg/dl), glucose (140.33-166.02 mg/ml),

Table 1 Immune effector activities in healthy pangas catfish, *Pangasius pangasius*

Immune parameters	Number of samples analysed	Range	Mean± SD
Serum lysozyme production (U/ml)	30	192.00–387.78	299.41±54.79
Ceruloplasmin activity (OD at 540 nm)	28	0.22–0.37	0.28±0.04
Anti-protease activity (inhibition %)	28	3.17–10.22	6.27±2.05
Myeloperoxidase activity (OD at 450 nm)	38	0.12–0.19	0.16±0.02
Respiratory oxidative burst activity (OD at 540 nm)	50	0.21–0.56	0.34±0.09
Nitric oxide production (µM)	30	130.00–510.00	316.84±118.87
In-vitro lymphocyte proliferation with concanavalin-A (OD at 540 nm)*	36	0.92–3.81	2.11±0.74
Without mitogen	36	1.09–3.60	2.06±0.71

* Specific immune response; SD: Standard deviation; OD: Optical density

Table 2 Levels of serum biomarkers and growth indicators in healthy pangas catfish, *Pangasius pangasius*

Serum biomarkers	Range	Mean±SD*
C-reactive protein (mg/L)	1.81-2.45	2.08±0.20
Cortisol (µg/dl)	30.15-41.94	36.10±6.12
Glucose (mg/dl)	140.33-166.02	150.76±6.12
Alanine aminotransferase (IU/L)	9.37-13.20	11.41±1.67
Aspartate aminotransferase (IU/L)	112.60-143.20	130.51±8.46
Lactate dehydrogenase (IU/L)	380.63-462.80	432.73±28.77
Creatinine (mg/L)	0.17-0.26	0.22±0.04
Insulin-like growth factor -1 (ng/ml)	8.30-10.64	9.32±0.63
Insulin-like growth factor binding protein-1 (ng/ml)	3.68-4.99	4.67±0.41

*Values are mean ±standard deviation (SD) of 12 samples

alanine aminotransferase (9.37-13.20 IU/L), aspartate aminotransferase (112.60-143.20 IU/L), lactate dehydrogenase (380.63-462.80 IU/L), creatinine (0.17-0.26 mg/ml), C-reactive protein (1.81-2.45 mg/L), insulin-like growth factor-1 (8.30-10.64 ng/ml), insulin-like growth factor binding protein-1 (3.68-4.99 ng/ml) are depicted in Table 2.

Discussion

The fish immune system is accountable for clearing the invading pathogens that cause infectious diseases (Swain et al. 2006). The results indicated variations in the immune effector activities among the different batches of catfish. The present study observed serum lysozyme levels of 192.00-387.78 U/ml with a mean of 299.41±54.79 U/ml in *P. pangasius*. Several studies reported varying levels of lysozyme in the plasma of different catfish species like 310.9±79.4-369±116.3 U/ml in *Silurus glanis* (Caruso et al. 2002), 5.31-7.23 µg/ml (=330-453 U/ml) in *Ictalurus punctatus* (Welker et al. 2007) and 207-440 U/ml in *Pangasianodon hypophthalmus* (Sirimanapong et al. 2014). Whilst the serum lysozyme levels were reported as 16.74±1.07 mg/ml (=705±60 U/ml) in *Clarias batrachus* (Kumari and Sahoo 2006) and 0.4-0.5 mg/L (=25-31 U/ml) in *C. gariepinus* (Kazuń and Siwicki 2013). The present results were, however, comparable to the levels recorded in the plasma of *P. hypophthalmus* (Sirimanapong et al. 2014). These results also indicated wide variations in the lysozyme levels among different catfish species. Likewise, Grinde et al. (1988) reported 5-10 folds variations in the lysozyme levels in 12 different fish species.

The ceruloplasmin activity of *P. pangasius* was in the range of 0.22-0.37 OD with a mean of 0.28±0.04 OD, which corroborates the observations (0.24±0.03-0.33±0.02 OD) of Adikesavalu et al. (2016). The anti-protease activity (% inhibition) of *P. pangasius* was very low (3.17-10.22; mean: 6.27±2.05) compared to the levels (60% as trypsin inhibition) noted in *P. hypophthalmus* (Sirimanapong et al. 2015) and *Labeo calbasu* (Mohanty et al. 2014). Likewise, the level of myeloperoxidase in *P. pangasius* (0.12-0.19 OD; mean: 0.16±0.02 OD) was markedly lower and distinct from the levels reported in other catfish species like *C. batrachus,* 0.50 OD (Kumari and Sahoo 2006) and *P. hypophthalmus* 0.72-0.78 OD (Sirimanapong et al. 2014). Furthermore, the myeloperoxidase levels of catfish were lower and different from those reported in carps (Sahoo et al. 2008; Mohanty et al. 2014).

In *P. pangasius*, the ROB activity and *in-vitro* NO production were observed to be 0.21-0.56 OD (0.34±0.09

OD) and 130.00-510.00 µM (316.84±118.87 µM), respectively. The variations in the ROB activity and NO production were about 2.6 and 3.9 folds, respectively among the different batches of catfish. Similarly, Dubey nee Pathak and Lal (2010) noted variations in NO concentrations in the serum of *C. batrachus* (8-50 µM), but the levels were lower than in *P. pangasius*. Earlier studies recorded the ROB activities of 0.427±0.017 OD at 610 nm in *I. punctatus* (Welker et al. 2007), 0.75 OD at 540 nm in *C. batrachus* (Kumari and Sahoo 2006) and 0.95 OD at 620 nm in *C. gariepinus* (Kazuń and Siwicki 2013). The results of the present study are, in general, lower than those reported for *I. punctatus* (Welker et al. 2007), *C. batrachus* (Kumari and Sahoo 2006) and *C. gariepinus* (Kazuń and Siwicki 2013) using similar methods of detection. In contrast, Sirimanapong et al. (2014) recorded low levels of ROB activity using $1x10^6$ cell/ml at 610 nm in *P. hypophthalmus*, i.e., 0.10-0.12 OD in the presence of NBT and 0.16-0.23 OD in the presence of NBT and phorbol myristate acetate (PMA). Also, the ROB activities (0.18–0.90 OD) reported in carps (Sahoo et al. 2005; Mohanty et al. 2014) were different from the *P. pangasius*, thus suggesting interspecies variations.

The *in-vitro* lymphocyte proliferation upon mitogen (Con-A) stimulation was observed to be in the range of 0.92-3.81 OD and a mean of 2.11±0.74 OD in *P. pangasius*, which corroborates Adikesavalu et al. (2016). The variations in the lymphocyte proliferation were about 4 folds among the different batches of catfish possibly attributed to the varying water temperature and the physiological status. On the other hand, the lymphocyte proliferation upon mitogen (Con-A) and lipopolysaccharide (LPS) stimulation in *C. gariepinus* was observed to be 0.43 and 0.30 OD at 620 nm, respectively (Kazuń and Siwicki 2013).

The CRP is an established diagnostic tool as early indicators of inflammation and plays beneficial roles in mediating the complex inflammatory response and seeking to restore homeostasis (Kodama et al. 2004; Giang et al. 2010). As seen in Table 2, in *P. pangasius,* the CRP levels were in the range of 1.81-2.45 mg/L, with a mean of 2.08±0.20 mg/L. In contrast, a low CRP level of 56 µg/L (=0.056 mg/L) was documented in *P. hypophthalmus* (Giang et al. 2010). Available reports suggested highly variable CRP contents in teleosts. For example, CRP levels of 1-2 µg/ml (=1-2 mg/L) in *Platichthys flesus* and *Scyliorhinus canicula*, 6.8 ng/ml - 5.3 mg/ml (=0.0068-5300 mg/L) in *Anguilla japonica*, 15-94 µg/ml (=15-94 mg/L) in *Oncorhynchus mykiss*, 55 µg/ml (=55 mg/L) in *Pleuronectes platessa,* 212 µg/ml (=212 mg/L) in *L. rohita,* 220 µg/ml (=220 mg/L) in *Channa punctatus* and 400 µg/ml (=400 mg/L) in *Mustelus canis* were documented (Giang et al. 2010). Discrepancies in sampling techniques, analytic methodology, and age, sex, species, the strain of fish selected and different stimuli may result in variable data, in which the level of serum CRP is either decreased or increased (Kodama et al. 2004).

Cortisol, the principal corticosteroid in teleostean, performs a functional role in mobilizing energy in fish. The levels of serum cortisol in *P. pangasius* were in the range of 30.15-41.94 µg/dl, with a mean of 36.10±6.12 µg/dl. The results are comparable to those of the observations of Adikesavalu et al. (2016) recorded in *P. pangasius* (30.20±2.04 µg/dl). Also, the results were relatively similar (34.93±2.28 µg/dl) to Shambhudan (2014) and markedly higher (25-27 ng/ml (=2.5-2.7 µg/dl)) than those recorded in *P. hypophthalmus* (Soltanian et al. 2014). In contrast, low levels of serum cortisol (7.13±3.42 µg/dl) were recorded in *I. punctatus (*Refaey and Li 2018) and *C. gariepinus* (10-12 ng/ml (=1.0-1.2 µg/dl)); Baßmann et al. 2017). These differences may be due to the individual variations in cortisol levels.

The serum glucose levels of *P. pangasius* ranged between 140.33 and 166.02 mg/dl with a mean of 150.76±6.12 mg/dl. The observed levels were marginally higher but comparable to the levels of 93.80±6.17-143.00±7.21 mg/dl (Shambhudan 2014) and 90-139 mg/dl (Yaghobi et al. 2015) recorded in *P. hypophthalmus.* Contrarily, the serum glucose levels of other catfish species were observed to be lower, i.e., 21.0±10.0-46.4±22.40 mg/dl (Ellsaesser and Clem 1987), 54.67±9.17 mg/dl in *I. punctatus* (Sánchez-Martínez et al. 2017) and 111.72±0.86 mg/dl in *C. gariepinus* (Mahmoud et al. 2012). *The* blood glucose levels in varying units like 2.0-3.0 mmol/L (=36-54 mg/dl) in *C. gariepinus* (Baßmann et al. 2017) and *I. punctatus* (Refaey and Li 2018) and 4.6-7.6 mmol/L (=82.8-136.8 mg/dl) in *P. hypophthalmus* (Galagarza et al. 2017) have also been documented.

In *P. pangasius,* the serum ALT levels ranged from 9.37 to 13.20 IU/L, with a mean of 11.41±1.67 IU/L. These levels were, more or less, in conformity with the observations of Yaghobi et al. (2015), who recorded ALT levels of 9.00±0.57 IU/L in *P. hypophthalmus*. On the other hand, lower ALT levels (4.50±0.70-6.66±1.15 IU/L) in *P. hypophthalmus* (Shambhudan 2014) and *I. punctatus,* 2.60±1.60-9.00±3.00 IU/L (Ellsaesser and Clem 1987) were documented. Mahmoud et al. (2012) recorded higher serum ALT levels of 40.92±0.42 IU/L in *C. gariepinus.* The serum AST levels of *P. pangasius* ranged between 112.60 and

143.20 IU/L with a mean of 130.51±8.46 IU/L, which corroborate to the median value (135 IU/L) noted in *P. hypophthalmus* (Galagarza et al. 2017). Ellsaesser and Clem (1987) also noted a marked fluctuation in serum AST levels (71.30±3.00-147.70±74.00 IU/L) in *I. punctatus*. In contrast, the AST levels recorded in *P. hypophthalmus* (105.83±0.70 IU/L; Shambhudan 2014) and *C. gariepinus* (40.92±0.42 IU/L; Mahmoud et al. 2012) were relatively lower than in *P. pangasius.* The serum LDH levels of *P. pangasius* ranged from 380.63 to 462.80 IU/L with a mean of 432.73±28.77 IU/L. The levels were almost similar (435.50±19.79 IU/L) to the observations of Shambhudan (2014) recorded in *P. hypophthalmus,* who also noted fluctuation in LDH levels among individuals of various feeding groups. In contrast, a wide range of serum LDH levels (176.90±160.80-496.90±281.10 IU/L) was documented in *I. punctatus* (Ellsaesser and Clem 1987). The serum creatinine levels of *P. pangasius* ranged between 0.17 and 0.26 mg/L with a mean of 0.22±0.04 mg/L. Contrarily, the reported creatinine levels in *P. hypophthalmus* were either higher (0.283±0.005-0.456±0.015 mg/dl (=2.83±0.05-4.56±0.15 mg/L); Shambhudan 2014) or lower (0-8 µmol/L (=0-0.9 mg/L); Galagarza et al. 2017) than in *P. pangasius*. The documented creatinine levels in other catfish were in the range of 0.11-0.37 mg/dl (=1.1-3.7 mg/L) serum in *C. gariepinus* (Mahmoud et al. 2012) and 0.3±0.1-0.7±0.4 mg/dl (=3.0±1.0-7.0±4.0 mg/L) serum (Ellsaesser and Clem 1987) and 0.39±0.32-0.53±0.21 mg/dl (=3.9±3.2-5.3±2.1 mg/L) plasma of *I. punctatus* (Aguirre-Guzman et al. 2016).

The levels of serum IGF-1 in *P. pangasius* were observed to be in the range of 8.30-10.64 ng/ml, with a mean of 9.32±0.63 ng/ml. Alike, Adikesavalu et al. (2016) reported IGF-1 levels of 8.33±0.10 ng/ml in *P. pangasius*. Nguyen (2015) noted IGF-1 levels of 19.07±4.48 ng/ml in *P. hypophthalmus*, which was about, above 2 times higher than the present study. In *I. punctatus*, Silverstein et al. (2000) documented serum IGF-1 levels of 4.19±0.36 ng/ml at 21.7°C and 5.39±0.28 ng/ml at 26.0°C. They also reported the serum IGF-1 levels in the range of 4-12 ng/ml in *I. punctatus* in varied conditions (Silverstein et al. 2000). In yellow catfish *Pelteobagrus fulvidraco*, though the levels were unquantified, the expression of IGF-I was positively correlated with feed intake (Qin et al. 2020). The available data on the serum IGF-1 levels of different teleosts varied markedly. For example, serum IGF-1 levels of 1.07±0.06-2.38±0.36 ng/ml in Indian major carps (Abraham et al. 2017) to as high as 400 ng/ml in transgenic coho salmon (Devlin et al. 2004) have been documented. It is well known that IGF-1 is extremely vital for the regulation of growth and cell functions through different signalling (Picha et al. 2012). Evidence suggested that serum IGF-1 levels in teleosts positively correlate with growth rates of individuals and could be used to evaluate the growth performance for aquaculture (Silverstein et al. 2000; Picha et al. 2012; Qin et al. 2020). The variations in IGF-1 level within and across fish species may be due to the presence of diverse endogenous and exogenous factors.

The IGFBP family of teleosts is poorly understood compared to the mammalian system (Garcia de la Serrana and Macqueen 2018) and described as a negative regulator of teleost growth (Kajimura et al. 2003). The levels of serum IGFBP-1 in *P. pangasius* were observed to be in the range of 3.68-4.99 ng/ml with a mean of 4.67±0.41 ng/ml, which corroborate Adikesavalu et al. (2016). In *Carassius auratus,* increasing levels of IGFBP-1 was reportedly restricting the IGF signalling by binding with free IGF-1 and diverting the limited energy resources away from growth and development (Chen et al. 2016). The IGFBP-1 was also found to be upregulated in fish during environmental stress and infection (Waagbø et al. 2017), which links growth to innate immunity, thus, potentially down-regulating the growth (Adikesavalu et al. 2016). An increase in the IGFBP-1 level may, therefore, harm the growth of catfish.

The current diagnostic clinical pathology tools are limited to a few species. The results of the present study indicated that the innate immune parameters and serum biomarkers of *P. pangasius* are different from other catfish species and teleosts. It provided the baseline values for some of the key non-specific (both humoral and cell-mediated) and specific immune parameters as well as serum biomarkers of *P. pangasius*, which could be used as indicators, to assess the health status of *P. pangasius*. Appreciably, these observations may provide the fundamental information and a better understanding of the immune effector activities and health status of this species during the stress, infections and therapy, and for developing immunoprophylactic and stress mitigation measures in commercial catfish aquaculture.

Conflict of interest The authors declare that they have no conflict of interest.

Authors contributions All authors contributed equally.

Compliance with ethical standards All applicable guidelines of the Committee for the Purpose of Control and Supervision of

Experiments on Animals (CPCSEA), Government of India, New Delhi and the University Ethical Committee were followed by the authors. All efforts were also made to minimize the suffering of the animals.

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