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Assessment of Intra-Ovarian Melatonin Levels and its Oxidative Stress Management with the Growth of the Ovary from Advanced Juvenile to Mature Adult Carp, *Catla catla*

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Abstract

The present study aimed at understanding the age-dependent intra-ovarian melatonin levels and their role in oxidative stress management in relation to the ovarian growth and development in the carp, *Catla catla*. Samples of ovaries were collected from different growth stages, *viz*. (i) advanced juvenile (AJv), BW >200 g to ≤300 g; (ii) preadult (PA), BW >300 g to ≤500 g; (iii) subadult (SA), BW >500 g to ≤1.00 kg; and (iv) adult (AD), BW >1 kg to ≤2.5 kg. Notably, in the current investigation the intra-ovarian melatonin concentrations, and levels/activities of intra-ovarian malondialdehyde (MDA), and different anti-oxidative agents including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GRd), glutathione S-transferase (GST), and reduced glutathione (GSH) in four different growth stages of carp, *Catla catla* were measured. Data analysis revealed significantly high MDA levels in PA carp, followed by lower values in AJv, that too associated with the lowest melatonin concentration in both AJv and PA carp. In contrast, SA carp exhibited the lowest MDA levels concomitantly with the highest melatonin levels, followed by slightly increased MDA levels and the highest melatonin concentrations in AD. Moreover, SOD, CAT, and GPx activities gradually increased from AJv to AD carp, with the highest activities in AD, moderate in SA, and lowest in AJv and PA carps. The results also demonstrated that GRd, GST, and GSH activities/levels were highest in AD carp, with no significant changes in AJv, PA, and SA carps. Following the correlation analysis and PCA, melatonin levels in the ovary and activities/levels of all anti-oxidative agents were negatively associated with intra-ovarian stress (MDA) but positively related to each other. However, according to the correlation test, GRd and GST activities did not correlate significantly with the GSH level. Overall, the observations suggest that early ovarian growth in AJv and PA carp experiences peak oxidative stress due to pubertal onset and development, impairing major antioxidant functions. However, subsequent ovarian growth and maturation enhance melatonin levels and other antioxidant functions, thereby reducing intra-ovarian oxidative stress.

Keywords: Antioxidants, Carp, Growth Stages, Intra-Ovarian Melatonin, Oxidative Stress, Pubertal Onset

1. Introduction

In most organisms, oxidative stress is produced due to various physiological activities, such as metabolic changes, somatic development[1,2], and variations in the season, including temperature fluctuations, levels of oxygen, and availability of food. Additionally, stress is also generated during gonadal development in fish[3-5]. Oxidative stress plays a critical role in physiological ageing[6], characterised by the accumulation of cellular and tissue changes detrimental to age-related health conditions[7]. In order to reduce oxidative stress, living organisms have developed various antioxidant mechanisms that decrease the production of Reactive Oxygen Species (ROS) and repair the resulting oxidative damage[8,9]. During the last few years, melatonin has emerged as a naturally produced antioxidant in any organism, which is essential for the mitigation of ovarian stress, particularly in fish reproduction[10,11]. Moreover, melatonin is a well-known antioxidant that has different

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therapeutic uses in health and agriculture. However, the early anti-oxidative defences involve glutathione, a non-enzymatic agent that mitigates ROS production via its scavenging function[12]. Glutathione (GSH), a vital antioxidant, operates within cells by reducing hydrogen peroxide (H_2O_2) and lipid peroxides (LPO), serving as a cofactor for GSH peroxidases. Additionally, it detoxifies electrophilic toxicants by functioning as a conjugant of Glutathione S-transferase (GST), regulates protein functioning, and manages nucleotide synthesis[13,14].

The aquatic organisms utilize the main anti-oxidative defence system including enzymes like Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), Glutathione reductase (GRd), and GST (second phase biotransformation enzyme) to detoxify ROS. SOD dismutases the superoxide radicals (O_2^-) into H_2O_2, subsequently generating water and oxygen with the combined function of CAT and GPx activities. Further, GSTs mainly function as phase II detoxification enzymes, safeguarding cells from internal and external alkylating agents, including those derived from xenobiotics[15]. These enzymes, located within cells, detoxify by catalysing the conjugation of glutathione (GSH) with endogenously found toxic by-products and environmental contaminants[16].

In numerous investigations, it has been observed that fish belonging to various ages exhibit distinct age-dependent regulation of antioxidants in plasma and other tissues, such as the kidney, liver, brain, gill, heart, *etc.*[17-20]. However, no study has yet been conducted to evaluate the antioxidant status in view of intra-ovarian melatonin levels and other anti-oxidative agents in the ovary of different growth stages of fish. Notably, ovaries are highly susceptible to environmental factors due to their intricate morphological structure and diverse physiological roles, affected by direct and uninterrupted exposure to rainfall and temperature under natural conditions[21-23]. Furthermore, the ovary, due to its follicular development and maturation, is mainly susceptible to the production of ROS, and to counteract this, it serves as one of the sources of melatonin[24,25] and as a repository of both enzymatic and non-enzymatic anti-oxidative agents, which reduce the generation of oxidative stress. Therefore, it is appropriate to examine the variations in the levels of intra-ovarian melatonin and levels/activities of intracellular stress (MDA) and various antioxidant agents (SOD, CAT, GPx, GRd, GST, and GSH) during the growth stages, *viz.*, advanced juvenile (AJv) (ovarian onset) to adult (AD) stages (matured ovary) of the carp, *Catla catla*, under natural photo-thermal conditions. Extensive research has been carried out on both humans and non-primate vertebrates, yielding inconsistent outcomes regarding the alterations in antioxidant enzyme activities that occur as a result of the ageing process[26]. Similarly, inconclusive reports are also found regarding the association of the activities/levels of anti-oxidative agents with different ages of the fish[4]. Indeed, extensive investigation should be conducted on various fish species to comprehend the alterations in the level of intra-ovarian melatonin, intracellular oxidative stress, and antioxidant enzyme activities in the ovary that occur during the fish's growth and ageing.

2. Materials and Methods

2.1 Chemicals and Reagents

Melatonin ELISA kits (Catalogue no. RE54021) were purchased from IBL International, Hamburg, Germany. Leupeptin hemisulphate, 2-thiobarbituric acid (TBA), molecular biology reagent water, hydrogen peroxide (H_2O_2), and hydrochloric acid (HCl) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trichloroacetic acid (TCA), citric acid, nitrobluetetrazolium (NBT), nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADPH), reduced glutathione (GSH), sodium hydroxide (NaOH), phenyl methyl sulphonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), 5, 5'-dithiobis (2-nitrobenzoate) (DTNB), 5-methylphenazinium methyl sulfate (PMS), o-phenylenediamine (OPD), 1-chloro-2, 4-dinitrobenzene (CDNB), glutathione oxidized (GSSG), and other commercially pure chemicals or reagents were obtained from SRL (Mumbai, India).

2.2 Care, Rearing, and Feeding of Fish

Carp specimens from four growth stages (i) advanced juvenile (AJv), (BW > 200 g to ≤ 300 g and BL ~ 240-260 mm); (ii) preadult (PA), (BW > 300 g to ≤ 500 g and BL ~ 260-300 mm); (iii) subadult (SA), (BW > 500 g to ≤ 1.0 kg and BL ~ 300-400 mm); and (iv) adult (AD), (BW > 1 kg to ≤ 2.5 kg and BL ~ 400-460 mm), were collected from the stock which was being maintained earlier by the Fisheries Research and Training Centre (FRTC)[27]. The physicochemical parameters of the pond water, including pH, total alkalinity, hardness, dissolved oxygen, carbon dioxide, and plankton abundance,

were thoroughly assessed and closely monitored at the appropriate levels throughout the period of study. However, in order to ensure the optimal health of the fish, regular liming and manuring activities, as well as supplementary feeding when necessary, were performed.

2.3 Ovary Sample Collection and Tissue Extract Preparation

The fish were subsequently anaesthetised using 2-phenoxyethanol before being euthanised through decapitation. A rapid dissection was performed to obtain the ovarian samples. In each growth stage, at least six female individuals were sacrificed to obtain ovary samples, which were collected and stored for further analysis. In brief, the paired ovary was made free from the extraneous tissues and fluids by soaking on blotting paper, and the weight of the ovary of the respective fish was noted. The process of preparing the 10% tissue homogenate involved mixing the tissue with 0.1 M phosphate buffer (pH 7), 0.1 mM PMSF, and 10 µM leupeptinhemisulfate in a glass homogeniser at 4°C. Following homogenization, the supernatant was obtained through centrifugation at 8000 × g for 10 min at 4°C, and it was then stored at -20°C until it was utilised for measuring the levels of ovarian melatonin and levels/activities of oxidative stress and various anti-oxidative agents. The protein concentration in the supernatant was determined by the Bradford method using BSA as a standard. For each sample, all analyses were performed in triplicate to ensure accuracy.

2.4 Estimation of Melatonin Concentrations in the Ovary of AJv to AD Carp

The supernatants of carp ovarian tissue were extracted, and melatonin concentration was estimated using a commercially available ELISA kit (Melatonin ELISA, IBL, Hamburg) according to the manufacturer's instructions[27-29]. The absorbance was taken at 405 nm in a Bio-Rad iMark Micro Plate Absorbance Reader. Briefly, the ovarian tissue homogenate (500 µL), standards (500 µL), and controls (500 µL) were extracted with 1 mL of methanol using a C18 reverse phase extraction column (catalog no. KEME761). Nitrogen was used to evaporate the methanol, and the dried extract was reconstituted with 150 µL of molecular biology-grade water. Each extracted sample (50 µL), standard (50 µL), and control (50 µL), prepared in duplicate, were incubated with melatonin biotin (50 µL) and melatonin antiserum (50 µL) in a microtiter plate for 14 hours at a temperature of 2-8°C. Following the incubation period, the solution was discarded, and the microtiter plate was washed three times with wash buffer. Subsequently, it was incubated with the enzyme conjugate (150 µL) at Room Temperature (RT). After two hours, the solution was again discarded, and the microtiter plate was washed three times with wash buffer. Thereafter, the PNPP (p-nitrophenyl phosphate) substrate was added, and the microtiter plate was incubated on an orbital plate shaker (Tarsons, Spinix-multiwell shaker, Model No. 3400) for 40 min at RT. Finally, after the addition of 50 µL PNPP stop solution, the absorbance was measured at 405 nm using a Bio-Rad iMark microplate absorbance reader (Model No. 1681135). The standard curve was established utilising the OD values (absorbance) of the standards, and the melatonin concentrations in the samples were subsequently determined from this standard curve, expressed as pg/g tissue. The Melatonin ELISA kit provides extraction columns, a microtiter plate, melatonin biotin, melatonin antiserum, an enzyme conjugate, melatonin standards and controls, a wash buffer, substrate solution, and a stop solution.

2.5 Measurement of the Levels of Malondialdehyde (MDA) in the Ovary of AJv to AD Carp

After centrifuging the ovarian tissue homogenates at 3000×g for 15 min, the supernatants were used to assess the level of MDA using the slightly modified TBARS (thiobarbituric acid reactive substances) assay, as described by Draper and Hadley (1990)[30]. The process entailed heating 1 mL of samples with TBA reagent (containing 20% trichloroacetic acid, 0.5% TBA, and 2.5 N HCl; 2 mL) for 20 minutes in a boiling water bath. After allowing the solution to cool, it was centrifuged at 500×g for 10 minutes, and the resulting precipitate was discarded. The absorbance at 532 nm of the supernatant of each sample was measured using a spectrophotometer (Shimadzu UV-1900i). An extinction coefficient of 1.56×10^5/Mcm was used in determining the MDA equivalents of the provided sample.

2.6 Measurement of the Activities/Levels of Different Anti-Oxidative Agents in the Ovary of AJv to AD Carp

2.6.1 Superoxide Dismutase (SOD)

The measurement of ovarian SOD activity was performed by a microplate reader (Bio-Rad iMark MicroPlate

Absorbance Reader) to access the absorbance at 560 nm[31]. Briefly, the protocol entailed transferring a 25 µL enzyme sample to a microtiter plate well that was filled with 200 µL of a freshly prepared 50 mM phosphate buffer (pH 7.4) containing 98 µM NADH, 62 µM NBT and 0.1 mM EDTA. To initiate the reaction, 25 µL of 33 µM PMS was added to 50 mM phosphate buffer at pH 7.4 that contained 0.1 mM EDTA. The substrates, PMS and NADH, were diluted serially to validate the assay. The changes in absorbance of the test samples that lacked substrates remained negligible.

2.6.2 Catalase (CAT)

The standard method described by Aebi (1984)[32] was employed to measure CAT activity in the ovarian tissue samples. Briefly, the procedure involved the addition of 40 µL of the enzyme sample to a cuvette containing 2 mM H_2O_2 in phosphate buffer, which was then immediately mixed. The absorbance was subsequently measured at 240 nm using a UV-Vis spectrophotometer (Shimadzu UV-1900i) for up to 90 s at intervals of 15 s. To validate the assay, the ovarian samples were treated with sodium azide, an inhibitor of CAT activity[33].

2.6.3 Glutathione Peroxidase (GPx)

Spectrophotometric methods were utilised to evaluate the activity of GPx[34]. Briefly, 100 µL of enzyme sample was combined with 1 mL of OPD in phosphate citrate buffer (pH 5.0), thoroughly mixed with 0.9 mL of 0.013% H_2O_2, and incubated at room temperature for 30 minutes. The absorbance at 492 nm was measured using a Shimadzu UV-1900i spectrophotometer in relation to a blank solution containing 100 µL of additional OPD solution instead of the sample. Under standard assay conditions, one unit of enzyme activity increases absorbance (A492) by 1.0; thereby, the enzyme activity was expressed in terms of units. To validate the accuracy of the assay, serial dilutions of OPD, the substrate for GPx, were used.

2.6.4 Glutathione Reductase (GRd)

The measurement of the ovarian GRd activity involved monitoring the NADPH oxidation, dependent on glutathione, in a reaction mixture that comprised 950 µL of 0.15 mM NADPH, 0.5 mM glutathione, and 3 mM $MgCl_2$ in 50 mM Tris (pH 7.5), along with 50 µL of extract, at a wavelength of 340 nm using the Shimadzu UV-1900i spectrophotometer[35]. Corrections were applied to the NADPH oxidation process in the absence of glutathione.

2.6.5 Glutathione S-Transferase (GST)

The measurement of GST activity involved the use of a standard method described by Habig *et al.* (1974)[36], which utilised CDNB (1 mM/L) and GSH (2.4 mM/L) as substrates. The absorbance was taken at 340 nm at a regular interval of 60 s for 5 min using a Shimadzu UV-1900i spectrophotometer. The enzymatic activity was expressed as U/mg protein, and the assay was validated by the serial dilution of the substrates (GSH and CDNB) to measure the optimal concentrations for the highest amount of GST activity.

2.6.6 Reduced Glutathione (GSH)

Ovarian GSH level was measured following the method described by Ellman[37]. The supernatant was mixed with 5% perchloric acid (100 µL) and then centrifuged at 800×g for 10 min at 4°C. After a 3 min incubation at room temperature, the reaction mixture (2 mL) comprising 100 µL of supernatant, 1.88 mL of 0.1 mol/L potassium phosphate buffer (pH 8.0), and 0.02 mL of 4% DTNB was measured for its absorbance at 412 nm using a Shimadzu UV-1900i spectrophotometer at a 60 s interval for 5 min.

2.7 Statistical Analysis

The means and standard error of means (SEM; n=6) were calculated and analyzed for the homoscedastic data of melatonin, MDA, and various anti-oxidative agents, including SOD, CAT, GPx, GRd, GST, and GSH in the ovaries of AJv to AD carp using one-way analysis of variance (ANOVA). The post-hoc Duncan's multiple range test was used to compare the mean values, with a significance threshold of $P< 0.05$[38]. All statistical analyses and data representation were conducted using MS Excel and SPSS.

3. Results

3.1 Measurement of the Ovarian Melatonin Levels

Data analysis revealed significant ($P<0.001$) age-dependent alterations in the ovarian melatonin levels from AJv to AD carp, in which AJv and PA carp showed the lowest melatonin levels. However, an increased level of ovarian melatonin was observed in the SA carp, followed by the maximum level in the AD (Figure 1).

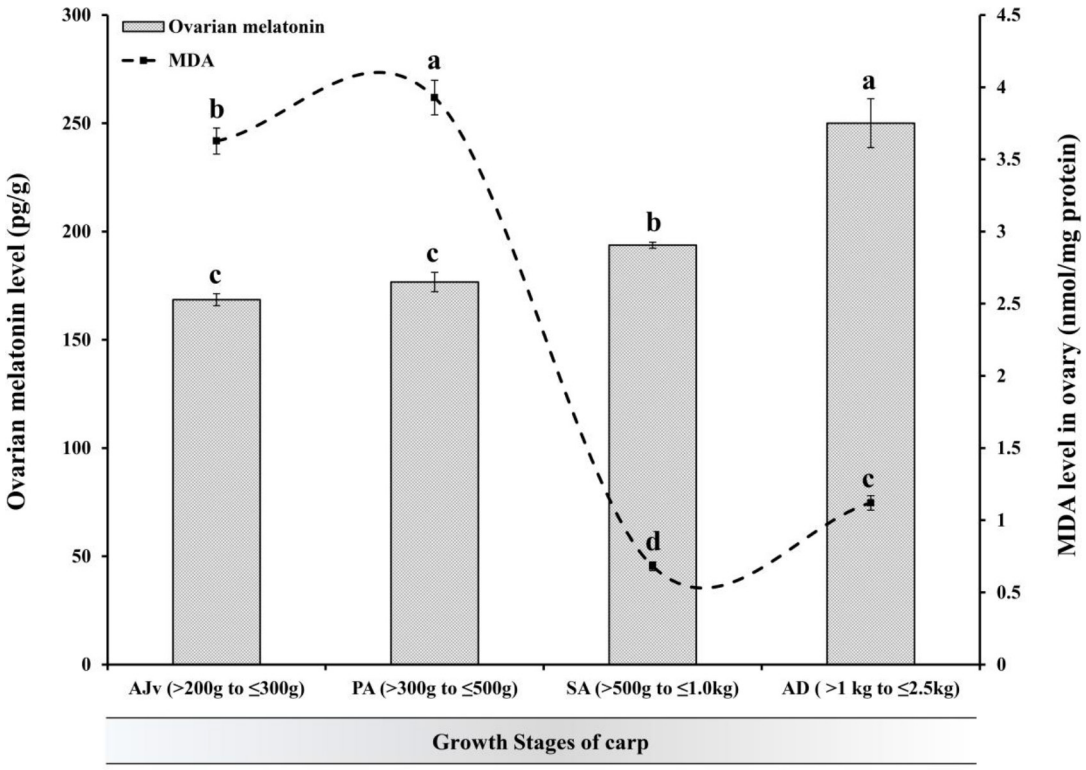


Figure 1. Graphical presentation of the data (mean ± SEM; n = 6) on ovarian melatonin level (in dark grey filled vertical bar) and malondialdehyde (MDA) level (in black dotted line) in four different growth stages of carp (AJv, PA, SA, and AD) fed a balanced diet at ~10:00 h for 15 days. Different letters on the mean values (P<0.05) show significant variation between growth stages within a parameter.

3.2 Measurement of the Ovarian MDA Level

The data analysis revealed statistically significant (P< 0.001) variations in ovarian MDA levels with age, particularly from the AJv to AD stage. A high level of MDA was detected in the ovary of AJv carp, which increases further in the PA stage, followed by a 4-5 fold decrease in the ovarian MDA levels in SA. In the AD carp ovary, though, there is a slight increase in MDA levels compared to SA, but they remained much lower compared to AJv and PA groups (Figure 1).

3.3 Measurement of the Activities/Levels of Ovarian Anti-Oxidative Agents

3.3.1 Measurement of the Ovarian SOD Activity

Data analysis revealed significant (P<0.001) age-dependent changes in the activity of SOD in the ovary from AJv to AD carp in which AJv and PA carp showed much-lowered activity. However, an increased activity of ovarian SOD was observed in the SA carp, followed by the maximum activity (6-7 fold increase) in the AD (Figure 2A).

3.3.2 Measurement of the Ovarian CAT Activity

Results showed significant (P< 0.001) growth stage-dependent variations in CAT activity in the ovary of SA and AD carp. The AJv and PA carp exhibited substantially reduced activity of ovarian CAT; however, the ovary of SA carp demonstrated significantly increased CAT activity, which was further increased almost 3-fold in the AD carp ovary (Figure 2B).

3.3.3 Measurement of the Ovarian GPx Activity

The analysed data exhibited a notable increase (P<0.001) in GPx activity in the ovary of SA to AD carp. While AJv and PA carp showed markedly lower GPx activity, the ovary of SA carp displayed an increase in GPx activity. However, compared to the SA carp, the AD carp ovary showed a 2-3 fold rise in GPx activity (Figure 2C).

3.3.4 Measurement of the Ovarian GRd Activity

The data analysis revealed AD carp ovary (P<0.001) to retain 2-3-fold increase of GRd activity compared to any of the earlier growth stages (SA, PA, AJv carp) (Figure 2D).

3.3.5 Measurement of the Ovarian GST Activity

The analysed data exhibited almost 2-fold (P<0.001) increased GST activity in the ovary of AD carp, compared to SA, PA, or AJv, where the ovary remains immature. Throughout the reproductively immature stages (AJv, PA, and SA) of carp, the GST activity in the ovary remained low (Figure 2E).

3.3.6 Measurement of the Level of Ovarian GSH

Results showed a significant (P<0.001) increase in the level of ovarian GSH in SA and AD carp. Notably, the levels of ovarian GSH remained low in the AJv and PA carp when the ovary remained immature and small in size (Figure 2F).

3.4 Pearson Correlation Coefficient Analysis and PCA among the Melatonin Levels, Intracellular Stress Marker (MDA), and Different Anti-Oxidative Agents (SOD, CAT, GPx, GRd, GST, and GSH) in the Ovary

The correlation between intracellular stress marker (MDA) and melatonin, as well as each of the anti-oxidative agents (SOD, CAT, GPx, GRd, GST, and GSH) in the ovary, was found to be negative from the AJv to AD stages of carp. In addition, positive correlations have been found between ovarian melatonin and all other anti-

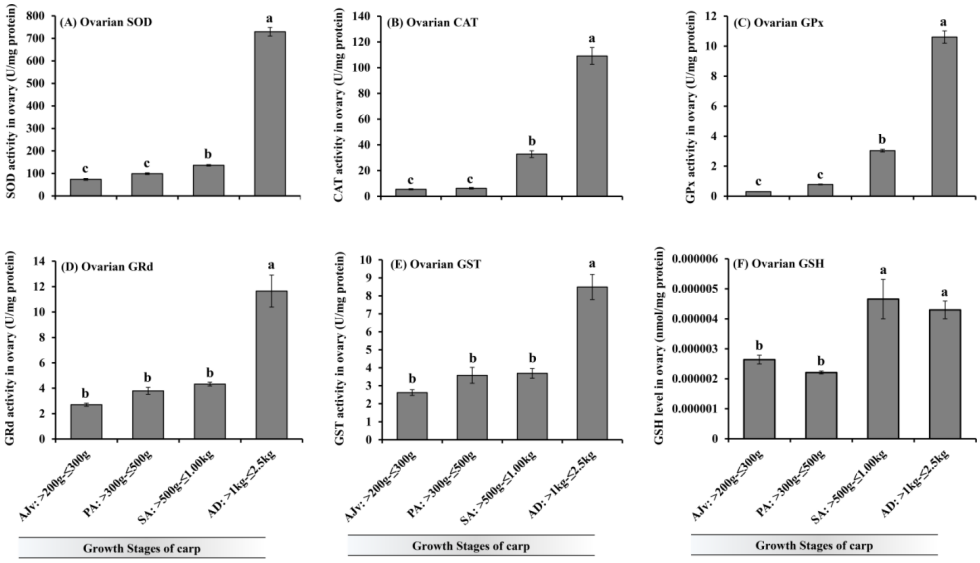


Figure 2. Graphical presentation of the data (mean ± SEM shown in vertical bars; n = 6) on **(A)** superoxide dismutase (SOD), **(B)** catalase (CAT), **(C)** glutathione peroxidase (GPx), **(D)** glutathione reductase (GRd), **(E)** glutathione S-transferase (GST) and **(F)** reduced glutathione (GSH) in four different growth stages of carp (AJv, PA, SA, and AD) fed a balanced diet at ~10:00 h for 15 days. Different letters on the mean values (P<0.05) show significant variation between growth stages within a parameter.

Table 1. Values of 'r' revealed from simple correlation coefficient analysis of the values of melatonin, malondialdehyde (MDA), and different anti-oxidative agents in the ovary of different growth stages of carp, *Catla catla*.

	Different redox parameters in the ovarian tissues						
	Ovarian melatonin	**MDA**	**SOD**	**CAT**	**GPx**	**GRd**	**GST**
	Advanced Juvenile (AJv) to Adult (AD)						
MDA	- 0.639[a]	-	-	-	-	-	-
SOD	+ 0.863[a]	- 0.537[b]	-	-	-	-	-
CAT	+ 0.910[a]	- 0.669[a]	+ 0.962[a]	-	-	-	-
GPx	+ 0.864[a]	- 0.669[a]	+ 0.979[a]	+ 0.977[a]	-	-	-
GRd	+ 0.893[a]	- 0.516[b]	+ 0.908[a]	+ 0.944[a]	+ 0.896[a]	-	-
GST	+ 0.726[a]	- 0.498[c]	+ 0.890[a]	+ 0.842[a]	+ 0.893[a]	+ 0.818[a]	-
GSH	+ 0.559[b]	- 0.756[a]	+ 0.387	+ 0.483[c]	- 0.466[c]	+ 0.359	+ 0.313

[a] P<0.001, [b] P<0.01, [c] P<0.05

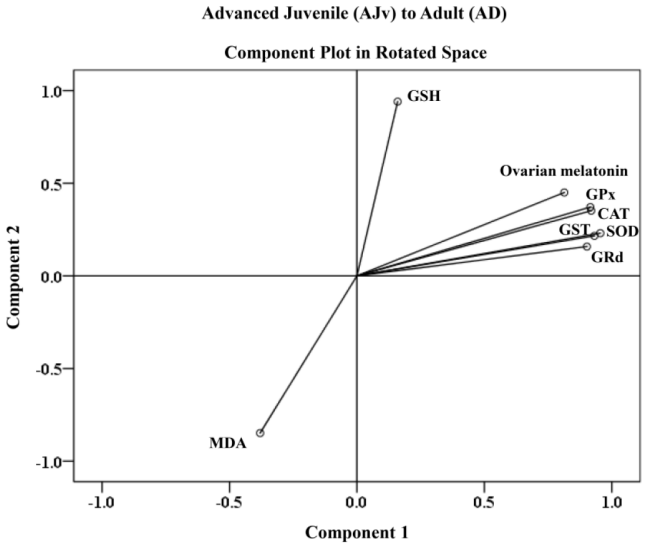


Figure 3. Principal Component Analysis (PCA) bi-plot for the data of ovarian melatonin levels, MDA levels, and activities/levels of different anti-oxidative agents (SOD, CAT, GPx, GRd, GST, and GSH) in the ovary of various growth stages (AJv, PA, SA, and AD) of carp, *Catla catla*.

oxidative agents; however, GRd and GST activity did not significantly correlate with the GSH level (Table 1).

Moreover, an equal number of principal components were identified by analyzing the PCA data of the ovarian melatonin level, MDA level, and the activity/level of SOD, CAT, GPx, GRd, GST, and GSH. The component matrix by extraction showed that all the parameters except the MDA were loaded with the same positive sign on PC1, accounting for 91.3% of the total variations (Figure 3).

4. Discussion

The present investigation reports novel findings for the first time, revealing that the levels of ovarian melatonin, intracellular oxidative stress, and the function of anti-oxidative agents exhibit substantial variations throughout the ovarian growth and developmental phases in carp species across the AJv to AD stages. The analysed data revealed that AJv carp exhibited high ovarian MDA levels, which were significantly elevated in the PA stage. However, the SA carp had the lowest value, followed by a slightly higher value in AD (Figure 1). Thus, it is plausible to suggest that the initiation of ovarian function (onset of puberty) and the subsequent development in the AJv and PA carp may contribute to the generation of ROS, potentially resulting in the highest level of intra-ovarian stress. On the contrary, further development and maturation of the carp ovary in the SA and AD carp significantly lowered the oxidative stress. These observations further indicate that varied ovarian growth and developmental status directly influence stress generation during different growth stages from AJv to AD. In a prior investigation conducted by Barcellos *et al.*[39] female catfish (*Rhamdia quelen*) revealed a heightened sensitivity to stress during ovarian onset and development, as opposed to adult females. This highlights the crucial function of puberty in regulating the Hypothalamo-Pituitary-Interrenal (HPI) axis in fish. Furthermore, according to the available data, a significant change in the fish's response to stress was observed as a result of the development of the neuroendocrine stress axis in the salmonid species. Based on the findings of Koakoski *et al.*[40], cortisol levels were detected earlier in juvenile fish than in adult fish following exposure to stress, suggesting an enhanced stress response in juvenile fish that also highlights the substantial role of age in fish[41]. Nonetheless, an elevation in blood cortisol levels induced by stress may take care of the oogenesis process, particularly by incorporating it into the yolk protein during vitellogenesis. In addition, cortisol has been found to play a significant role in regulating the function of maturation-inducing hormone during the process of oocyte maturation and ovulation in fish; specifically, the increased activity of 11β-hydroxysteroid dehydrogenase 2 in the theca and/or granulosa cells has been identified as the primary protective mechanism to prevent the accumulation of excess cortisol in the developing fish oocyte[42]. Moreover, a recent investigation by Parolini *et al.*[20] reported that age had a substantial influence on LPO levels in both the gills and the liver, exhibiting a decrease in LPO with advancing age. Based on the prior observations, the current study may suggest that AJv carp may exhibit greater susceptibility to environmental stress factors, ultimately resulting in increased oxidative stress within the ovary under natural conditions compared to the AD stage.

However, ovarian melatonin levels showed an inverse relation with the levels of MDA during different ovarian growth and developmental stages from AJv to AD, in which AJv and PA carp exhibited the lowest levels of ovarian melatonin, and the SA and AD showed the highest levels (Figure 1). These observations lead to some similar clues to the previous observation by Hasan *et al.*,[10] in which seasonal variations of MDA levels showed a significant peak during the post-spawning phase and

decreased gradually thereafter, with the minimum value in the spawning phase of an annual cycle in carp *Catla catla*. This indicates that ovarian oxidative stress is high during post-spawning and low during spawning, which may deal with the similar observations from AJv to AD stages of carp, *Catla catla*. However, following correlation and PCA, the presence of a substantial negative correlation between melatonin and MDA levels in the ovary suggests that ovarian melatonin may play an antioxidant role (Table 1 and Figure 3). Notably, a recent study by Sutradhar *et al.*[27], revealed that the gut of AJv carp exhibited the lowest melatonin levels along with the highest oxidative stress levels with the onset and development of the ovary, which further demonstrates the inverse relation between melatonin and MDA. However, several investigations demonstrated that increased local and denovo synthesis of ovarian melatonin may enhance the oocyte maturation in AD[25,43,44]. Based on these observations, it may be suggested that the amine hormone melatonin may be a key regulating factor in reducing intra-ovarian oxidative stress by acting as an antioxidant as well as activating the different antioxidant functions during oocyte maturation in AD carp[11,45,46].

Moreover, the generation of differential stress response by the ovary of various growth stages of carp from AJv to AD is mitigated by the activities/levels of different anti-oxidative agents such as SOD, CAT, GPx, GRd, GST, and GSH. Results indicated the gradual progression in the activities of SOD, CAT, and GPx from AJv to AD carp in which the highest activities were seen in AD carp, moderate in the SA, and the lowest activities in AJv and PA carps (Figures 2A, B, C). The observations indicate that pubertal onset and development possibly contribute to the maximum production of intracellular stress, resulting in lowered activities of SOD, CAT, and GPx in the ovary. These results were also found to be similar to the observations of intra-ovarian melatonin levels, in which both the AJv and PA carp exhibited the lowest levels of melatonin. These observations were supported by the correlation and PCA, which indicated a negative relationship between intra-ovarian stress and each of the anti-oxidative agents in different growth stages of carp (Table 1 and Figure 3). In this context, an investigation on carp growth stage-dependent gut melatonin profile has reported that ovarian appearance in the AJv carp is associated with the lowest level of gut melatonin,[27] which may be linked with the reduced activities of SOD, CAT, and GPx in the ovary due to its regulatory action in the management of stress. However, the further growth and maturation of the ovary in the adult carp may be associated with heightened activities of SOD, CAT, and GPx, which suggests stage-specific anti-oxidative function during different growth stages of carp from AJv to AD. The current findings also suggest that carp ovaries under the initial growth stages may generate excess free radicals and superoxide anions, which would contribute to an increased production of H_2O_2. This, in turn, may inactivate the intra-ovarian enzyme activity in the AJv and PA stages of carp, *Catla catla*. These outcomes align with the previous findings in several fish species[19,47], exhibiting lower enzymatic activity in the early ages of fish compared to the later stages. However, during the later stages of ovarian growth and maturation in the SA and AD carps, the increased activity of SOD was found to dismutase the superoxide anion into H_2O_2 which is broken down into water and oxygen by the increased activities of CAT and GPx, thereby considered as the primary anti-oxidative system that maintains the balanced oxidative status within the ovary. In view of these observations, melatonin in the carp ovary may act as a key factor to augment the primary antioxidant system by stimulating SOD, CAT, and GPx activities to reduce ovarian oxidative stress in the later growth stages of carp, *Catla catla* (SA and AD)[10,11]. Moreover, a study conducted by Parolini *et al.*[20] on brown trout (*Salmo trutta*) revealed notable alterations in enzyme activities that aid in managing stress in gill and liver tissues under natural environmental conditions. However, the observations of this study differ from the current findings in that an age-dependent increase in GPx activity was observed in both gill and liver tissues. Additionally, the study found a decrease in liver SOD activity with increasing age, while no trend was observed in gill tissue. Furthermore, no significant impact of age was observed on CAT activity in both gill and liver tissues. The results of the present investigation are also consistent with a prior study that demonstrated enhanced activity of CAT and GPx in the gills of older rainbow trout compared to younger ones[19]. However, prior to this study, no such data were available for the ovarian tissue during various growth stages of carp. Another report by Nayak *et al.*[48] showed increased activity of liver GPx with the progression of age in the freshwater murrel (*Channa punctatus*). In a similar manner, an age-related increase in the activities of GPx and CAT was observed in the plasma and erythrocytes of the Adriatic sturgeon (*Acipenser naccarii*), as reported by Sanz *et al.*[49].

The present investigation also reports approximately 2-3 fold increased GRd and GST activity in the ovary of AD carp, compared to the early growth stages (SA, or PA, or AJv), where the ovary remains immature (Figures 1D, E). However, ovarian GSH retains its maximum levels in the SA carp and maintains up to the ovary of the AD carp (Figure 1F). Based on these findings, it may be concluded that the ovary of AD carp possesses a higher capacity to detoxify xenobiotic substances than the early growth stages of carp. However, melatonin may be a key factor in enhancing xenobiotic detoxification by elevating the levels and activities of GSH, GRd, and GST in the ovary of fish[50]. The present findings align with the results of a previous study conducted on rainbow trout by Otto and Moon[19], which demonstrated that glutathione concentrations and GST activities were highest in tissues from older age groups compared to younger age groups of trout. In this study, it was found that high levels of glutathione and its associated enzyme activities in the AD carp may serve as a reservoir for the fish ovaries, ultimately leading to enhanced oocyte development and fertilization[51]. The current study further reports that GRd and GST activities in the ovary are not significantly correlated with the GSH level following the correlation coefficient analysis and PCA (Table 1 and Figure 3). Notably, GSH directly interacts with ROS and also functions by donating electrons to certain peroxidases, including GPx. Moreover, GSSG, a product of oxidation, is either reconverted through electron transfer from NADPH by the activity of GRd or extruded from the cells. Therefore, GRd plays an indirect role in safeguarding cells against intra-ovarian oxidative stress[52]. In addition, higher peroxidative activities of ovarian GST are essential for regulating stress during the maturation of fish ovaries.[51] However, in the context of xenobiotic detoxification within the ovary under natural environmental conditions, the role of GST may be doubtful due to its lack of association with the intra-ovarian GSH level following the correlation and PCA analysis (Table 1 and Figure 3). According to the existing literature, this is the first report on the measurement of melatonin levels, the levels/activities of oxidative stress, and various anti-oxidative agents in the ovary of different growth stages of carp from AJv to AD. However, it is essential to obtain conclusive data from numerous experimental studies in order to comprehend the precise mechanism of action of intra-ovarian melatonin and its other antioxidant functions, which is crucial for maintaining optimal ovarian health in different growth stages of the carp, *Catla catla*.

5. Conclusions

The present study provides the first-ever evidence that underlines the functional interplay among intra-ovarian oxidative stress, melatonin levels, and different anti-oxidative agents during the various growth and developmental stages of the ovary from AJv to AD carp, *Catla catla*. According to these reports, there appear to be changes in the levels of intra-ovarian melatonin, MDA as well as the activities/levels of anti-oxidative agents, such as SOD, CAT, GPx, GRd, GST, and GSH, throughout the growth stages of carp. Notably, as ovarian growth and development in carp are intricately linked with the various phases of an annual reproductive cycle, it is arguably reasonable to comprehend the relation between growth stage-dependent ovarian melatonin and MDA levels and their different anti-oxidative agents with the various reproductive seasons. In this context, a study[10] reported maximum levels of carp intra-ovarian stress (MDA) during the post-spawning phase, which gradually decreased up to spawning with the minimum ovarian stress. With these intra-ovarian stress responses, carp ovaries experienced higher melatonin concentrations as well as enhanced antioxidant function (particularly SOD, CAT, and GPx activities) in the spawning followed by gradually declined activities in the pre-spawning and preparatory phases. These findings may correspond to the current observations noted in the AJv, PA, SA, and AD stages of the carp ovary. In addition, it[47] was reported that seasonal changes significantly alter the antioxidant function in different ages of fish. Therefore, it may be inferred that varied ovarian growth and development status in different growth stages of carp showed a connection with the different phases of an annual reproductive cycle. Nonetheless, the observations revealed that early ovarian growth and development in AJv and PA carp experience maximum oxidative stress due to pubertal changes, which results in the inactivation of major antioxidant functions. In contrast, further development and maturation of carp ovaries augment intra-ovarian melatonin levels and other antioxidant functions, which ultimately can reduce intra-ovarian oxidative stress.

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