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1 **ABSTRACT**

2 The oxidative action of copper on different trout *Oncorhynchus mykiss* erythrocyte components was
3 studied. The results indicate that:

- 4 - cupric ions differently influence the oxidative status of two trout hemoglobin components I
5 and IV (HbI and HbIV) having different structural and functional properties;
- 6 - Reactive oxygen species (ROS) production associated with hemoglobins autoxidation is not
7 influenced by the presence of copper sulphate;
- 8 - the susceptibility to hemolysis increases in the presence of copper only when the erythrocyte
9 suspension is incubated in air; the effect of copper is almost absent for carbon monoxide-
10 saturated erythrocyte suspensions;
- 11 - DNA damage due to copper was not observed in our experimental conditions.

12 The data obtained are important for the analysis of the environmental risks produced by copper on
13 fish.

14

15

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17 **KEYWORDS:** Copper, trout erythrocyte, hemoglobin, hemolysis, DNA damage

1 1. INTRODUCTION

2 Copper is an essential trace metal for living organisms and its role as cofactor for crucial enzymes
3 (cytochrome c oxidase, superoxide dismutase, ceruloplasmin, monooxygenases) has been well
4 established. Most of these enzymes catalyze oxidation/reduction reactions that have molecular
5 oxygen as co-substrate. However, if copper is present at relatively high concentrations in the
6 environment, toxicity to aquatic organisms can occur (Winger et al., 2000; Marcano et al., 1996;
7 Roche and Boge, 1996). In aquaculture systems, copper is regularly used in the form of copper
8 sulfate as a therapeutic agent and it is widely used to control alga blooms and aquatic macrophyte
9 infestations (Boyd and Massaut, 1999).

10 There is no doubt that exposure to excess copper can damage cells and organs because copper ion
11 and its complexes can induce formation of reactive oxygen species that can damage biomolecules
12 such as lipids, proteins and DNA. Copper toxicity at the cellular level depends on the permeability
13 of cell membranes to copper ions and on its participation in redox reactions because it easily cycles
14 between the cuprous and cupric state.



16 This paper focuses on the “in vitro” effects of copper ions on oxidative damage in trout
17 *Oncorhynchus mykiss* erythrocytes. The effect of copper ions on the oxidation of trout hemoglobin
18 components either as purified proteins or in the whole hemolysate was examined. It is worth
19 recalling that contrary to mammals and birds, a multiplicity of hemoglobin components is generally
20 present in erythrocytes of fish. In the case of nucleated erythrocytes from trout *Oncorhynchus*
21 *mykiss*, four different hemoglobin components are present; they are characterized by functional
22 differences which have been correlated with different physiological roles (Brunori, 1975). Trout
23 hemoglobin components according to their anionic mobility have been called HbI, HbII, HbIII and
24 HbIV. These hemoglobins are prone to oxidation and this property permits to follow “in vitro” the
25 autoxidation process over a relatively short time. The production of reactive oxygen species during
26 the hemoglobin oxidation was also evaluated. The measurements regarding purified hemoglobins

1 were performed on HbI and HbIV. These two trout hemoglobin components represent, respectively,
2 about 20 and 60% of the whole pigment present inside trout erythrocytes and have very different
3 oxygen binding properties (Brunori, 1975). The mechanism of copper-induced met-hemoglobin
4 formation has been described in detail for human hemoglobin solutions (Bonaventura et al., 1999)
5 but this is not well understood for fish hemoglobins that have different structures. Furthermore, the
6 influence of copper on the extent of hemolysis and on the susceptibility of DNA to oxidative
7 damage was assessed.

1 2. MATERIALS AND METHODS

2 All the reagents used were of analytical grade.

3 2.1 Samples

4 Red blood cells were obtained from *Oncorhynchus mykiss* trout, coming from the fish farm “Eredi
5 Rossi Silvio” Sefro MC, Italy. The fish were kept in tanks containing water from Scarsito river, a
6 tributary of the Potenza river, and fed with commercial fish food obtained from Hendrix S.p.A
7 (Mozzecane, VR, Italy). Blood was extracted by puncturing the lateral tail vein. After washing with
8 isotonic medium at pH 7.8 (0.1 M phosphate buffer 0.1 M NaCl, 0.2 % citrate, 1 mM EDTA) the
9 cells were suspended in the desired isotonic buffer.

10 2.2 Separation of hemoglobin components and carbon monoxide hemoglobin (HbCO) formation

11 Separation of the various hemoglobins, as described previously (Binotti et al., 1971) was carried out
12 by column chromatography on DEAE-Sephadex A-50; their homogeneity was tested by
13 electrophoresis.

14 Experiments involving carbon monoxide(CO) hemoglobin were carried out after exposure of the
15 oxygenated derivative to a weak vacuum by using a rotary vane pump and then to pure CO gas. The
16 vacuum, performed for a few seconds, permits the removal of part of the oxygen thus making the
17 formation of CO hemoglobin easier (Hb affinity for CO is greater than that for O₂ by about 250-
18 fold).

19 2.3 Ferric hemoglobin (Met-Hb) formation and hemoglobin precipitation

20 The rate of met-Hb formation in the hemolyzates and in two electrophoretically distinct
21 hemoglobin components called hemoglobin trout I and IV (HbI and HbIV) was followed in a Cary
22 219 spectrophotometer in the visible region; reference value (i.e. complete oxidation) was estimated
23 by addition of ferricyanide. Absorbance at 700 nm was followed as an index of turbidity to monitor
24 the onset of hemoglobin precipitation.

1 *2.4 Reactive oxygen species production during Hb oxidation*

2 ROS production associated with Hb autoxidation was evaluated by monitoring the oxidation of
3 non-fluorescent dichlorofluorescein (DCFH) to fluorescent DCF.

4 A solution of 1 mg ml⁻¹ of HbI or HbIV was incubated for 1 hour in phosphate buffer 50 mM pH
5 7.5 at different temperatures and in the presence of 0.036 M DCFH and of 10 or 100 μ M CuSO₄.
6 DCFH oxidation was measured fluorimetrically on these solutions at excitation and emission
7 wavelengths of 498 nm and 520 nm respectively.

8 *2.5 Hemolysis*

9 In order to evaluate the hemolysis rate, the erythrocytes were suspended in isotonic medium at pH
10 6.3 at 35°C. The degree of hemolysis was determined spectrophotometrically at 540 nm as
11 previously described (19) either in the presence or absence of the desired amount of copper sulfate.
12 In particular, hemolysis was determined as $100 \times A / 10 \times A^* \times 100\%$ where A is the optical density of
13 Hb present in the supernatant after centrifugation of red cell suspension and A* \times 100% is the optical
14 density of the red cell suspension after complete lysis with 10 volumes of distilled water at zero
15 time incubation.

16 *2.6 Single-cell gel electrophoresis (comet assay).*

17 The “comet” assay was performed on trout erythrocytes (1 10^6 cells ml⁻¹) incubated in a pH 6,3
18 isotonic buffer at 35°C for 1 hr in the presence and absence (control) of 50 μ M copper sulfate. After
19 incubation, the erythrocytes were suspended in 0.7% low melting agarose in phosphate buffer saline
20 (PBS) and pipetted on microscope slides pre-coated with a layer of 1% normal melting agarose. The
21 agarose with the cell suspension was allowed to set on the pre-coated slides at 4°C for 10 min.
22 Subsequently, another top layer of 0.7% low melting agarose was added and allowed to set at 4°C
23 for 10 min. The slides were then immersed in lysis solution (1% sodium *n*-lauroyl-sarcosinate, 2.5
24 M NaCl, 100 mM Na₂EDTA, 10 mM Tris HCl pH 10, 1% Triton X-100 and 10% dimethyl
25 sulfoxide for 1h at 4°C in the dark, in order to lyse the embedded cells and to permit DNA

1 unfolding. After incubation in lysis solution, slides were exposed to alkaline buffer (1 mM
2 Na₂EDTA, 300 mM NaOH buffer, pH >13) for 20 min; in this condition RNA is completely
3 degraded. The slides were subjected to 20 min electrophoresis at 25 V in the same alkaline buffer
4 and finally washed with 0.4 M Tris HCl buffer (pH 7.5) to neutralize excess alkali and to remove
5 detergents before staining with ethidium bromide (2 g ml⁻¹).

6 Cells were examined with an Axioskop 2 plus microscope (Carl Zeiss, Germany) equipped with an
7 excitation filter of 515-560 nm and a magnification of x20. Imaging was performed using a
8 specialized analysis system (“Metasystem” Altussheim, Germany) to determine tail moment (TM),
9 tail length (TL) and tail intensity (TI), parameters correlated with the degree of DNA damage in
10 each single cell. Experiments were performed three times and data (at least 150 scores per sample)
11 are the mean values plus/minus the standard error of the mean (SEM). Statistical analysis was
12 performed using the student t-test and differences were regarded as statistically significant when p
13 values were < 0.05.

1 3. RESULTS

2 3.1 *Met-Hb rate formation in hemolysates*

3 Figure 1 is one representative set of tracings from a total of at least three experiments and shows the
4 time course of hemoglobin autoxidation during incubation at 35°C in 50 mM phosphate buffer, pH
5 7.5, of trout hemolysate in the absence and presence of 10⁻⁶ M CuSO₄. As can be observed, the
6 presence of 10⁻⁶ M CuSO₄ increases the met-Hb formation rate; after two hours of incubation, the
7 level of met-Hb was ~30% and ~25% in the presence and absence of copper sulfate, respectively.
8 The statistical analysis of the three experiments give a p value <0.05.

9 3.2 *Met-Hb rate formation in purified hemoglobin components HbI and HbIV*

10 Figure 2 shows the time course of HbI autoxidation when the protein was incubated at 35°C and pH
11 7.5. The presence of high amounts of CuSO₄ (up to 100⁻⁶ M) does not influence the HbI oxidation
12 rate with respect to the control (HbI alone without CuSO₄) during our incubation time.

13 It was not possible to perform similar experiments with HbIV because this hemoglobin component
14 is less stable and tends to precipitate under these experimental conditions. The presence of copper
15 sulfate accelerates the protein denaturation rate. A similar behaviour was also observed at lower
16 incubation temperatures (30°C).

17 3.3 *Reactive oxygen species production during Hbs incubation*

18 ROS production associated with Hb autoxidation was followed by means of fluorescence deriving
19 from DCFH oxidation. Figures 3 and 4 show the time course of DCF fluorescence generated during
20 one hour incubation of HbI or HbIV followed at different temperatures. After one hour of HbI
21 incubation at 20°C and 25°C there is no ROS formation and this is in agreement with the absence of
22 HbI oxidation. When the HbI solution is incubated for one hour at 35°C ROS formation due to the
23 partial oxidation of the protein was observed (Fig. 3). Similar experiments were performed with
24 HbIV (the highest temperature was 30°C) (Fig. 4). A slight ROS production was present also at
25 25°C which increased at 30°C. For both hemoglobins the level of ROS production was not

1 influenced by the presence of copper sulfate. It is possible to avoid the formation of met-Hb by
2 adding carbon-monoxide (CO) before incubation. Experiments performed with HbCO did not show
3 ROS formation at any temperature (data not shown). This behaviour is due to the stability versus
4 the oxidation of the carbomonoxy-derivative; in other words, HbCO does not lead to the ferric form
5 during incubation.

6 *3.4 Hemolysis rate*

7 By varying pH and temperature, it is possible for trout erythrocytes suspended in isotonic medium
8 to follow “in vitro” the haemolytic event over a relatively short time (Falcioni et al., 1987); the
9 hemolytic process is characterized by the presence of a long lag phase. The influence of a fixed
10 amount (50 μ M) of copper sulfate on the rate of haemolysis in trout erythrocytes during incubation
11 in isotonic buffer at 35°C and pH 6.3 was investigated and reported in Fig. 5. The figure is one
12 representative set of tracings from a total of at least three experiments. The susceptibility to
13 haemolysis increases in the presence of copper only when the erythrocyte suspension was incubated
14 in air; a dose-dependent effect of copper was absent for CO-saturated erythrocyte suspensions
15 exposed to air under identical conditions. The half time ($t/2$) of haemolysis (expressed as the time
16 necessary for 50% haemolysis to occur) and determined from Fig. 5 is 160 min, 186 min, 148 min
17 and 183 min for red blood cells (RBC) suspension in air, RBC suspension with CO, RBC
18 suspension in air with 50 μ M CuSO₄ and RBC suspension with CO and 50 μ M CuSO₄, respectively.
19 The statistical analysis of the three experiments give a p value <0.05.

20 *3.5 Comet assay*

21 The “comet assay” or single-cell gel electrophoresis was performed on trout erythrocyte suspension
22 to explore if the copper salt under study influenced the DNA status in these nucleated cells. This
23 test has become increasingly popular for the measurement of DNA damage in individual cells and
24 consists of embedding cells in agarose, followed by lysis, electrophoresis and staining to visualize
25 DNA damage using fluorescence microscopy. Cells with increased DNA damage display an
26 increased migration of genetic material in the direction of the electrophoresis. The extent of DNA

1 damage is quantified by measuring the displacement of the genetic material between the cell
2 nucleus (“comet head”) and the resulting “tail”. The parameters used as an index of DNA damage
3 are tail length, tail intensity and tail moment and they are calculated by computerized image
4 analysis. The “comet assay” was performed on trout erythrocyte suspensions incubated at pH 6.3
5 and 35°C for 60 min in the presence of 50 M copper sulfate. Figure 6 clearly shows that all the
6 considered comet parameters (tail length, tail intensity and tail moment) increased after one hour.
7 The increase for tail length and tail moment was independent of the presence of copper sulfate in
8 the incubation medium; a slight increase was instead measured for tail intensity.

1 4. DISCUSSION

2 It is well known that copper can induce damage to cells through radical-formation (Valko et al.,
3 2005; Gaetke and Chow, 2003). Furthermore it is important to remember that copper, together with
4 iron and selenium, control the activity of those enzymes involved in the defence mechanisms
5 against reactive oxygen species (superoxide dismutase, catalase and glutathione peroxidase). The
6 results reported here clearly show that the presence of copper can influence the redox state of HbIV
7 and disrupts the plasma membrane. However, under our experimental conditions DNA damage due
8 to the presence of copper was not observed, except for a slight increase in tail intensity.

9 There is evidence that copper catalyzes human oxyhemoglobin oxidation also at very low
10 concentrations (Rifkind, 1974; Winterbourn and Carrel, 1997) while other metal ions have no
11 significant effect even at high concentrations. The copper induced met-Hb formation mechanism
12 has been described in detail for human hemoglobin (Bonaventura et al., 1999). It is probable that
13 ferric hemoglobin formation, in general, involves an electron transfer from Fe^{++} to Cu^{++} as
14 suggested for human hemoglobin (Bonaventura et al., 1999). Our results on the effect of copper on
15 autoxidation of trout hemoglobins clearly show that the effect of the metal is different in the two
16 hemoglobin fractions HbI and HbIV that have different aminoacid sequences, and as a
17 consequence, different tridimensional structures and functional properties (Barra et al. 1983;
18 Petruzzelli et al., 1989).

19 The involvement of the sulphhydryl group in -93 position in hemoglobin autoxidation has been
20 reported in the literature (Rifkind, 1972). The effect of copper on Hb oxidation during the
21 hemolysate incubation reported in Fig.1 could imply that the hemoglobin component oxidized is
22 HbIV and not HbI. This is due to the fact that HbI in the presence of copper at the highest
23 concentration and in the same experimental conditions was not oxidized.

24 It is worth pointing out that the autoxidation of oxygenated Hbs can be followed by the
25 transformation of the oxidized molecule (high-spin Fe^{3+}) into a species absorbing as a low-spin Fe^{3+}

1 compound, that is a hemichrome which tends to precipitate. The overall process may be described
2 according to the following scheme:

3 HbO_2 — met Hb — hemicrome — precipitate

4 The presence of different antioxidant enzymes (superoxide dismutase, catalase and glutathione
5 peroxidase) in the hemolysate could reduce the oxidation rate of HbIV and thus avoiding the
6 formation of HbIV precipitate.

7 The conversion of oxyhemoglobin into met-Hb is associated with superoxide production (Misra and
8 Fridovich, 1972; Brunori et al., 1975) and thereby of products such as H_2O_2 or hydroxyl radical,
9 which can derive from $\text{O}_2^{\cdot-}$ itself.

10 We have observed that the presence of copper sulfate does not influence the level of ROS during 1
11 hr of HbI or HbIV incubation (Figs.3-4). This result could be due to the direct interaction between
12 heme-iron and copper or to the participation of copper in redox reactions involving ROS.

13 The presence of Cu^{++} in the trout erythrocyte suspension influences the hemolytic process (Fig.5)
14 when the cells are exposed to air. The effect of Cu^{++} is absent when the experiment was performed
15 with CO-saturated erythrocytes; the presence of CO leads to insignificant hemoglobin auto-
16 oxidation over the same time range. These data appear to indicate that the effect of Cu^{++} on lysis is
17 also correlated with hemoglobin oxidation. The hemolytic event is a complex process due to more
18 than one type of biochemical damage. The difference in the rate of hemolysis between red blood
19 cells saturated with either CO or O_2 was due to the consequences following hemoglobin oxidation
20 (Falcioni et al., 1987). Recently, Labieniec et al. (Labieniec et al., 2009) found that incubation with
21 copper of marine fish erythrocytes from *Dicentrarchus labrax* increased hemolysis of cells; a
22 significant decrease in the hemoglobin content and red blood cells after exposure of fish *Channa*
23 *punctatus* to sublethal concentration of copper sulphate has been reported by Singh et al. (Singh et
24 al., 2008). On the membrane surface as well as at the cytosol level, copper ions are involved in
25 various redox reactions that lead to an increased radical production followed by oxidative damage
26 and lipid bilayer destruction. Cupric but not cuprous ions are responsible for met-Hb formation that

1 can contribute to the hemolytic event. The reduction of cupric to cuprous ion can occur at the
2 membrane surface level (Bogdanova et al., 1999) by the involvement of sulphhydryl groups. Both
3 cupric and cuprous ions can interact with transport systems outside and inside the membrane
4 leading to its perturbation (Bogdanova et al., 2002).

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1 **5. CONCLUSIONS**

2 In conclusion, the environmental risks from the use of copper compounds should be evaluated in
3 order to control biological damage to fish. The results here presented indicated that an eventual “in
4 vivo” alteration following copper exposure could induce plasma membrane perturbation with a
5 mechanism involving met-hemoglobin formation.

6

7

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1 **FIGURE CAPTIONS**

2

3 **Fig. 1.** Time course of Hb autoxidation in trout hemolysate containing Hb (1 mg ml^{-1}) during
4 incubation at 35°C in phosphate buffer 50 mM pH 7.5 in the presence and in the absence of 10 M
5 CuSO_4 . Data are from one representative set of tracings from a total of three experiments.

6 (\diamond) control; (\bullet) $+10 \text{ M CuSO}_4$;

7

8 **Fig. 2.** Time course of 1 mg ml^{-1} HbI autoxidation during incubation at 35°C in phosphate buffer 50
9 mM pH 7.5 in the presence and in the absence of 10 and 100 M CuSO_4 . Data are from one
10 representative set of tracings from a total of three experiments

11 (\bullet) control; (\square) $+10 \text{ M CuSO}_4$; (\blacktriangle) $+100 \text{ M CuSO}_4$

12

13 **Fig. 3.** Time course of DCFH (0.036 M) fluorescence generated during 1 hour incubation of HbI (1
14 mg ml^{-1}) in phosphate buffer 50 mM pH 7.5 at 20°C (Panel A), at 25°C (Panel B) and at 35°C
15 (Panel C) in the presence and absence of 10 and 100 M CuSO_4 . Data are from one representative
16 set of tracings from a total of three experiments.

17 (\blacklozenge) Phosphate buffer +DCFH

18 (\blacksquare) Phosphate buffer $+100 \text{ M CuSO}_4$ +DCFH

19 (\blacktriangle) Phosphate buffer +HbI+DCFH

20 (\circ) Phosphate buffer +HbI+ 10 M CuSO_4 +DCFH

21 ($*$) Phosphate buffer +HbI+ 100 M CuSO_4 +DCFH

22

23 **Fig. 4.** Time course of DCFH (0.036 M) fluorescence generated during 1 hour incubation of HbIV
24 (1 mg ml^{-1}) in phosphate buffer 50 mM pH 7.5 at 20°C (Panel A), at 25°C (Panel B) and at 30°C

1 (Panel C) in the presence and absence of 10 and 100 μM CuSO_4 . Data are from one representative
2 set of tracings from a total of three experiments.

3 (◆) Phosphate buffer +DCFH

4 (■) Phosphate buffer +100 μM CuSO_4 +DCFH

5 (▲) Phosphate buffer +HbIV+DCFH

6 (O) Phosphate buffer +HbIV+10 μM CuSO_4 +DCFH

7 (*) Phosphate buffer +HbIV+100 μM CuSO_4 +DCFH

8

9 **Fig. 5** Time course of hemolysis of trout erythrocyte suspensions ($\text{Hb} = 30 \text{ mg ml}^{-1}$) incubated in
10 isotonic buffer pH 6.3 and at 35°C in presence and absence of carbon monoxide and 50 μM CuSO_4 .

11 Data are from one representative set of tracings from a total of three experiments.

12 (◆) control; (▲) + 50 μM CuSO_4 ; (◇) control CO; (△) CO+ 50 μM CuSO_4

13

14 **Fig.6.** Tail length (A), Tail intensity (B) and Tail moment (C) comet parameter (mean \pm s.e.m)
15 measured on trout erythrocyte suspension incubated in isotonic buffer at pH 6.3 and at 35°C for 1
16 hour in the presence and in the absence of carbon monoxide and 50 μM CuSO_4 .

17 * $p < 0.05$ compared to control 1hr.











