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Research Article

# Micro- and Intermediate Filaments of the Testis of African Catfish (*Clarias gariepinus*) Treated with a Sub Lethal Dose of Carbendazim

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**Summary:** This study highlighted the effect of Carbendazim on the testicular micro and intermediate filaments adult male African catfish (*Clarias gariepinus*). Previous studies related to carbendazim toxicity in fish have been limited to mortality patterns and degree of sensitivity across species. Literature on actual pathology in fish is scanty. The fish were exposed to a pre-determined sub-lethal concentration (1.4 mg/L) of Carbendazim for seven and fourteen days, 10 fish were sedated by cold shock, and sacrificed on days seven and 14. Another untreated group (control) were sacrificed at the same periods. The testes were harvested and weighed. Testicular actin microfilament, cytokeratin, desmin and vimentin intermediate filaments were determined using standard immunohistochemistry protocols. Variations in the intensity and pattern of immuno-expression of the testicular actin, cytokeratin, desmin and vimentin were significant in a phase dependent manner with day 14 being more pronounced. Immunohistochemical features of degenerated and necrotic germinal and Sertoli cells in the treated group, with loss of wire-mesh network which supported the mature germinal cells in the testicular lumen were also observed. A sub-lethal dose of carbendazim exposure for either seven or 14 days, induced deliterous changes in the testicular micro- and intermediate filaments, of the African catfish. This portends a reduction in the male reproductive potentials of the exposed species and resultant negative impact on production.

**Keywords:** *Carbendazim, Clarias gariepinus, Histomorphology, Immunohistochemistry, microfilaments, intermediate filaments*

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## INTRODUCTION

Intermediate filaments are composed of a family, assembled from a large number of proteins that share related structural and sequence features. In epidermal cells, they feature more frequently than microfilaments or microtubules (Lodish, 2000). Intermediate filaments exist as structures/ layers adjacent to cellular and nuclear membranes, where they form network of associations and interactions with other cytoskeletal structures and receptors on the cell membrane ( Kobielak and Fuchs, 2004). In addition, intermediate filaments form extensive, organised structural system that connects the nuclear envelope to the plasma membrane (Djaball, 1999). Some intermediate filaments are found oriented parallel to the cell surface, while others are spread, scattered in the cytosol; together they form an underling framework with respect to the shape and resilience of the cell (Goldman *et al*., 1996).

There is also an existing interconnectivity between the filaments of adjacent cells. Distribution of forces, enhanced by this connectivity provides strength and the entire epithelial structure. The close proximity and connection of filaments with the associated plasma membranes suggest that their principal function is to mechanically support the plasma membrane of cells and the extracellular matrix, structurally reinforce and organize cells into tissues (Lodish, 2000). In spite of the fact that intermediate filaments arose from a vast assemblage of proteins , existing in a dynamic relationship as polymers, they have a more stable structure among the cellular structural proteins. By virtue of these, they are more readily observed intact in cells after tissue processing (Parry and Steinert, 1992; Herrmann and Aebi, 2016; Jones *et al*., 2017).

Actin filaments, also called microfilaments are cytoskeletal proteins with the greatest distribution in cells. The filaments are fairly abundant around the plasma membrane. They provide the foundation of mechanical support, cell shape, and cell kinetics at the surface, thereby enabling acts of migration, phagocytosis, and mitosis (Cooper, 2000). Within the cells they form three-dimensional networks, having properties of semisolid gels. The whole structural array of actin filaments and their relationships within cells are regulated by a variety of actin-binding

proteins, which are critical components of the actin cytoskeleton. Total protein constituents of muscle cells are approximately 20% actin filaments, it is about 5 to 10% of cellular protein in other types of eukaryotic cells (Cooper, 2000).

Cytokeratins are intermediate filaments generally found in the epithelia that line internal body cavities. They are closely related though distinguished from other keratin that forms the "hard" epidermal stratified squamous epithelium hat forms specialised structures of the skin. They can be used as important anatomical markers of normal and abnormal cell differentiation (Oliveira, 2005).

Vimentin, which is the most widely distributed of non-keratin intermediate filaments is typically expressed in, endothelial cells and leukocytes, some epithelial cells, and fibroblasts. The filaments form vital support of cell membranes as well as keep the cellular organelles in situ. Vimentin filaments are frequently seen in parallel network with microtubules. Desmin filaments are, compared to others, much limited in distribution. They are found as sarcomeres stabilizers in contracting muscle cells. Desmin filaments maintain muscle integrity, without them, there would be distruption of the muscle architecture and alignment.

The intermediate filaments are well expressed in the different compartments of the testicular structure and contribute well to the functional output. Their expression is also a useful feature in pathologic conditions (Rogatsch *et al*.,1996) i.e. in heightened expression in tumours and altered arrangement in tissue disruptive events seen in challenges with an environmental toxicant (Cheng, 2014).

Carbendazim (methyl 2-benzimidazole carbamate) is one of the most commonly used systemic pesticides. It is a widely used systemic fungicide with both chemoprotective and chemotherapeutic actions against a wide range of fungi in various vegetables and fruit trees (Mohapatra and Lekha, 2016). The fungicide is implicated as a persistent water pollutant (Fernandez *et al*.,2001; Cuppen *et al*., 2008). Carbendazim dressed fields could lead to contamination of ponds, water ways located near fields where it had been applied ( Aina *et al*., 2016). Also, there is the strong possibility carbendazim -contamination of the aquatic environment; earthen ponds, communal rivers related to farms through industrial effluents and farm equipment used in the application of the fungicide. Crops protected with Carbendazim based products end up as part of the raw materials for commercial production of fish feed (Aina *et al*., 2016.

One can infer from all these that degenerative and cellular distruptive conditions likely found in Carbendazim-associated tissue toxicity could negatively impact the architectural milieu of microfilaments and intermediate filament cytoskeleton in cells and tissues. Studies that related environmental toxicity in fish species appears to invest more in mortality pattern, bioaccumulation, respiratory components as well as the vital organs, there are fewer information on the effects on reproductive system.
This present study therefore aims at investigating the presence, expression and the histomorphology of Cytokeratin, Actin, Desmin and Vinmectin filaments in the testicular tissue of the matured African catfish exposed to a sub lethal dose of Carbendazim.

## MATERIALS AND METHODS

Testicular tissues obtained from all the exprimental groups of the adult male catfish (C. gariepinus) from control untreated seven day (MW1) , 14 day (MW2) , carbendazim treated seven day (MC1) and 14 day (MC2) groups were fixed in 10% Neutral Buffered Formalin for 48 hours. Fixed tissues were processed routinely for paraffin embedding technique as described by An *et al.,(* 2003).

### The immunostaining technique

The immunostaining technique was performed on 5µm-thick testicular sections, using a LSAB-plus kit (Dakocytomation, Denmark) as previously described in our earlier work ( Aina *et al*., 2019) . The empirical assessment of the intensities of vimentin, desmin, cytokeratin and actin immunostaining were done. The results were scored as absent (-), weak (+), moderate (++) and strong (+++) , relative to the positive control samples for each intermediate filament and actin microfilament

## RESULTS

Actin filament was weakly expressed in the testicular capsule and intertubular connective tissue. The cytoplasm of the maturing germinal cells and the cytoplasm of the Leydig cells reacted with a moderate intensity in all the groups. The carbendazim treated groups did not significantly differ in localization and intensity of the actin filaments, compared with the control (untreated groups). (Tables 1 and 2 ; Figures 1a and 1b)

Desmin was weakly expressed in the testicular capsule and the connecting interstitium, but strongly expressed in the sertoli cell cytoplasmic and its extensions which formed the cysts. There is a weak expression in the lumina mesh work that appears to suspend the mature spermatozoa. (Tables 1 and 2; Figure 3a).

The desmin staining accentuated the pathology of the testis seen at day 14 of carbendazim treatment as clumps of the luminal meshwork and desmin stained cellular debris can be seen clearly (Figure 3b)

**Table 1:**
Summary of the immunohistochemical localization of the intermediate filaments; cytokeratin, vimentin, desmin and the microfilament-actin in the testis of Carbendazim exposed and Control (unexposed) mature African Catfish at 7th day of Experiment

| Cell/Tissue stained | Actin | | Desmin | | Cytokeratin | | Vimentin | |
|---|---|---|---|---|---|---|---|---|
| | Carb | Control | Carb | Control | Carb | Control | Carb | Control |
| Testicular Capsule | + | + | + | + | + | + | -- | -- |
| Testicular interstitium | + | + | + | + | - | - | -- | -- |
| Basement membrane | -- | -- | -- | -- | +++ | +++ | -- | -- |
| Immature Germinal cells | ++ | ++ | -- | -- | -- | -- | -- | -- |
| Mature spermatozoa | -- | -- | -- | -- | -- | -- | -- | -- |
| Sertoli Cell Cytoplasm | -- | -- | +++ | +++ | + | + | +++ | +++ |
| Germinal Cysts | + | + | +++ | +++ | +++ | +++ | + | + |
| Luminal Meshwork | -- | -- | + | + | -- | -- | -- | -- |
| Leydig cells | | ++ | | -- | | +++ | | -- |

*a* disrupted; b* Free floating cells in the lumina*
*Intensities of immunostaining : --, absent; +, weak; ++, moderate;*

**Table 2:**
Summary of the immunohistochemical localization of the intermediate filaments; cytokeratin, vimentin, desmin and the microfilament-actin in the testis of Carbendazim exposed and Control (unexposed) mature African Catfish at 14th day of Experiment

| Cell/Tissue stained | Actin | | Desmin | | Cytokeratin | | Vimentin | |
|---|---|---|---|---|---|---|---|---|
| | Carb | Control | Carb | Control | Carb | Control | Carb | Control |
| Testicular Capsule | + | + | + | + | + | + | -- | -- |
| Testicular interstitium | + | + | + | + | - | - | -- | -- |
| Basement membrane | -- | -- | -- | -- | +++ | +++ | -- | -- |
| Immature Germinal cells | ++ | ++ | -- | -- | -- | -- | -- | -- |
| Mature spermatozoa | -- | -- | -- | -- | -- | -- | -- | -- |
| Sertoli Cell Cytoplasm | -- | -- | +++ | +++ | + | + | +++ | +++ |
| Germinal Cysts | + | + | +++ | +++ | +++ | +++ | + | + |
| Luminal Meshwork | -- | -- | + | + | -- | -- | -- | -- |
| Leydig cells | ++ | ++ | -- | -- | +++ | +++ | -- | -- |

*a* Disruptions accentuated by the presence of deeply stained clumps in the lumina*
*b* Free floating cells in the lumina Intensities of immunostaining : --, absent; +, weak; ++, moderate; +++, s*

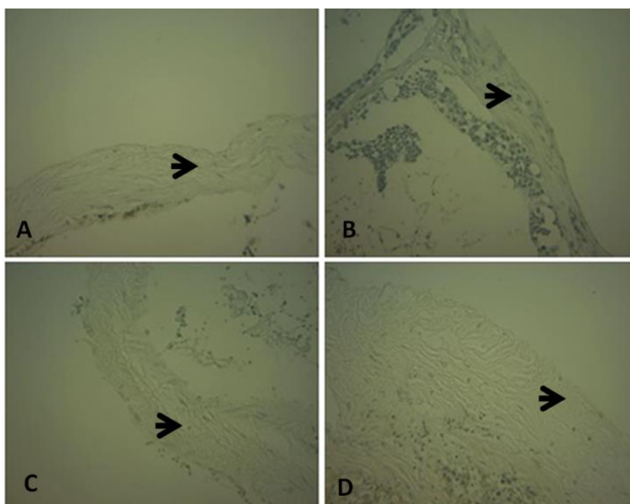


**Figure 1a:**
Weak expression of Actin filaments in at the capsule (arrows) of groups A : 7th day contol; B: 7th day carbendazim treated; C: 14th day contol; D: 14th day carbendazim treated

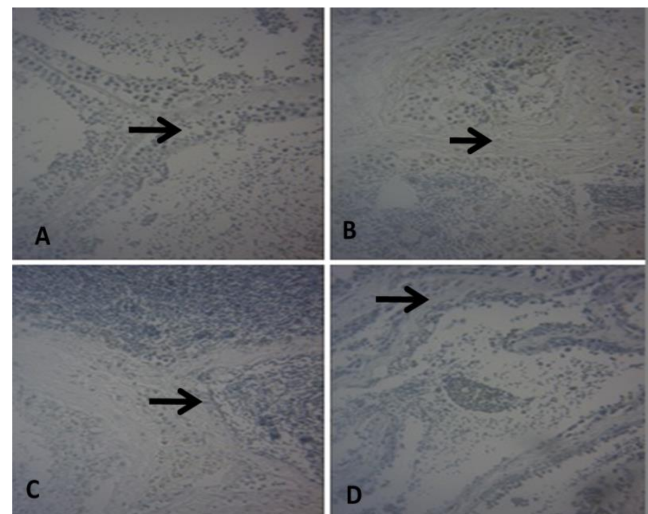


**Figure 1b**: Localisation of actin filaments in at the intertubular connective tissue (arrows) of groups A : 7th day contol; B: 7th day carbendazim treated; C: 14th day contol; D: 14th day carbendazim treated

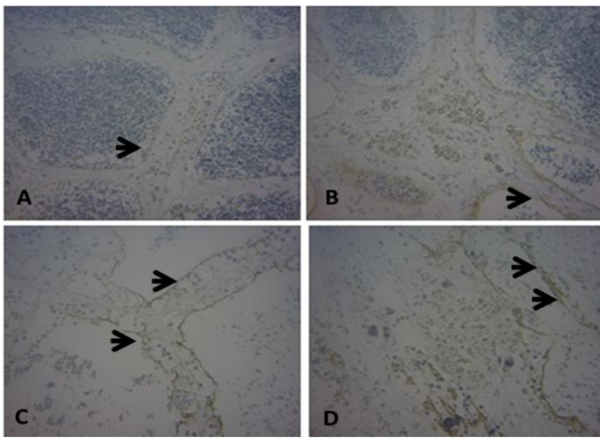

**Figure 2a**:
Intensity and localisation of cytokeratin filaments in at the basement membrane (arrows) of groups A : 7th day contol; B: 7th day carbendazim treated; C: 14th day contol; D: 14th day carbendazim treated

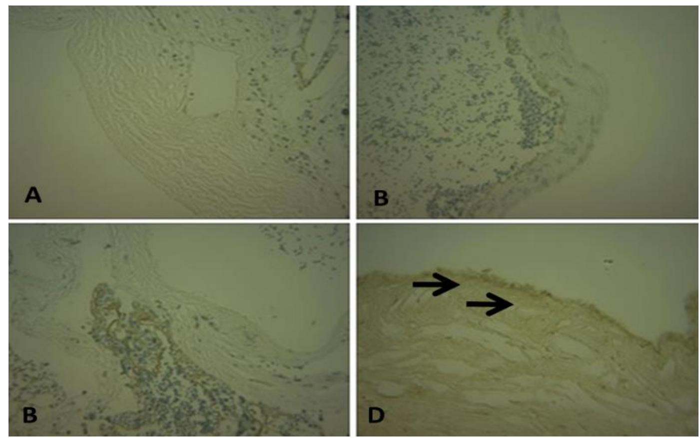

**Figure 2b:**
Intensity and localisation of cytokeratin filaments in at the testicular capsule (arrows) of group D: 14th day carbendazim treated. Groups A: 7th day contol; B: 7th day carbendazim treated; C: 14th day contol had poor cytokeratin

Cytokeratin filament is strongly the expressed in the basement membrane, germinal cysts and moderately in the leydig cells. In both the 7th and 14th day of carbendazim treatment, cytokeratin stained debris were free floating in the lumina while the cysts are greatly disrupted. Though there was a weak reaction at the capsule for the control groups, there was a relatively strong expression of cytokeratin in the capsule at the 14th day Carbendazim treatment. (Tables 1 and 2; Figures 2a and 2b).

Vimentin expression was seen strongly in the sertoli cell cytoplasm and slightly in its cysts forming cytoplasm. The spread of staining appears to follow the relatively pattern of disruption and scanty presence of sertoli cells in the treatment groups, especially the $14^{th}$ day carbendazim treated group. (Tables 1 and 2; Figures 4a and 4b).

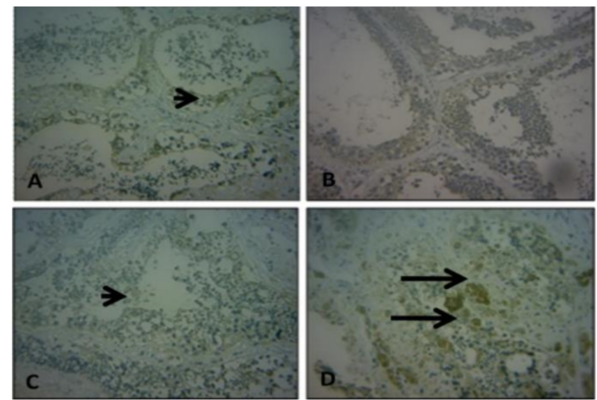

**Figure 3b:**
Intensity and localisation of cytokeratin filaments in at the testes of Groups A: 7th day contol; B: 7th day carbendazim treated; C: 14th day contol ; Group D: 14th day carbendazim treated. Desmin immunostaining. strongly expressed in the sertoli cell cytoplasmic and its extensions which formed the cysts ( short arrows). There is a weak expression in the lumina mesh work that appears to suspend the mature spermatozoa. The desmin staining accentuated the pathology of the testis seen at day 14 of carbendazim treatment as clumps of the luminal meshwork and desmin stained cellular debris can be seen clearly (long arrows)

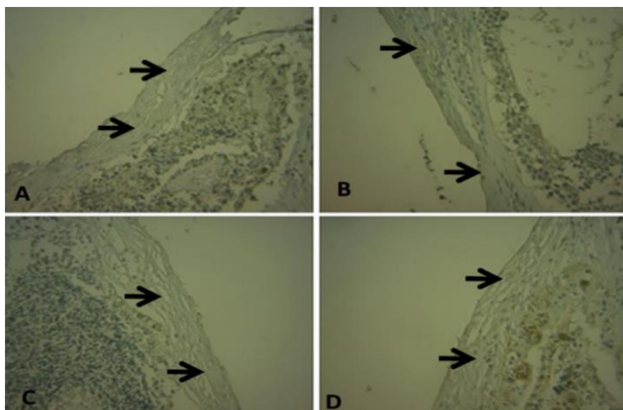

**Figure 3a**:
Intensity and localisation of desmin filaments showing weak expression at the testicular capsule (arrows) of . Groups A: 7th day contol; B: 7th day carbendazim treated; C: 14th day contol ; group D: 14th day carbendazim treated

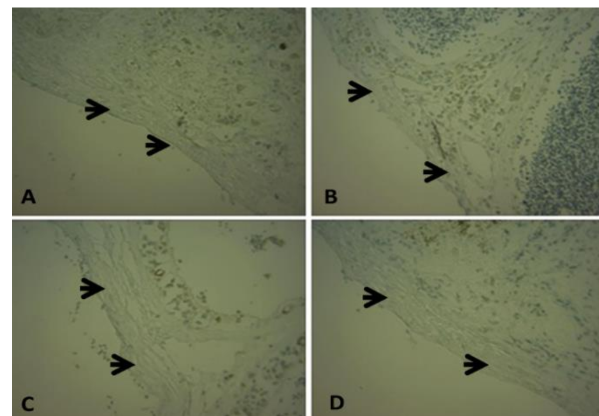

**Figure 4a**:
Intensity and localisation of Vimentin filaments showing weak expression at the testicular capsule (arrows) of . Groups A: 7th day contol; B: 7th day carbendazim treated; C: 14th day contol ; group D: 14th day carbendazim treated.

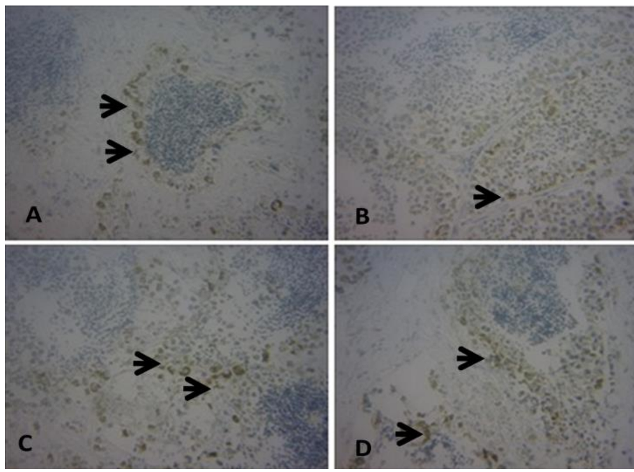


**Figure 4b**:
Intensity and localisation of Vimentin filaments showing strong expression (arrows) of the sertoli cells . Groups A: 7th day contol; C: 14th day contol , ; Groups B: 7th day carbendazim treated and group D: 14th day carbendazim treated also had strong sertoli expression which appears scattered in the lumen.

## DISCUSSION

Significant adverse effects of exposure to potentially toxic chemicals in the environment have been a well-recognised subject of study (Vighi and Villa, 2013). Tools that study the same adverse effects by sub lethal doses of the same chemicals would bring out a greater importance along this line, as drugs that have been confirmed non-poisonous at a given dose may actually carry a plethora of previously unseen effects at the same dose in species of concern. Moreover, in studies that determined lethal concentration of potential environmental toxins, most times, the basis of determination is somewhat limited to mortality pattern (Palawski and Knowles, 1986; Oruc, 2010; Rico *et al*., 2001)

The results present significant phase dependent variations in the intensity of immuno-expression of the testicular actin, cytokeratin, desmin and vimentin with day 14 being more pronounced. In a similar way, the extent of disruption of the filament meshwork is also higher with the later phase (day 14). Immunohistochemical features of degenerated and necrotic germinal and Sertoli cells in the treated group, with loss of wire-mesh network which supported the mature germinal cells in the testicular lumen were also observed. This further corroborates the earlier study that implicates Carbendadim in distruption of microtubule assembly (Winder, 2001)

Immunoexpression of the various cytoskeletal proteins helped to highlight the histomorphological alteration that could be associated with carbendazim exposure. The importance of this immunohistochemical study is not only in the degree of expression of target structures , but also the accentuation of tissue destruction beyond what is generally observed under routine H&E methods. This implies an added advantage of immunohistochemistry, not only as quantification of expression of proteins, but also enhancing tool for the structural features observed in routine H&E methods

The effects seen in the study such as loss in the cytoskeletal framework could be responsible for disruption of microtubule assembly which has been previously reported as a feature of Carbendazim exposure (Lim and Miller,1997; Winder *et al*., 2001).

A sound reproductive system is a cogent factor to the success of the aquatic species in the struggle for survival between pathogens and targets/hosts. The immunohistochemical tool applied for actin filament, desmin, cytokeratin and vimentin microfilaments helped to highlight to a large extent the structure detail of testicular disruption associated with Carbendazim in African Catfish. Simple immunohistochemical techniques therefore could expand and enhance the knowledge base of the pathogenesis of ecotoxicological agents on fish tissues determined through routine H&E methods.

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