BIOCELL
2022 46(4): 881-884

Tech Science Press

Study of cytoskeleton from microscopic point of view: Our experience

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Key words: cytoskeleton, Environment, Immunocytochemistry, Pathological condition

Abstract: The manuscript deals with our studies and experiences in the assessment of cytoskeleton in different cellular models and situations. The immunofluorescent study of several cytoskeletal proteins was relevant in the evaluation of a therapy for osteoarthritis, in case of alkaptonuria and in testing the efficacy of docetaxel in neuroblastoma cancer cells leading to apoptosis. A relevant part of our experience focus on the study of cytoskeleton in seminiferous epithelium and spermatozoa, identifying alterations affecting blood-testis barrier after a silver nanoparticle treatment, chromosomal segregation in case of varicocele, sperm motility and diagnosing systematic sperm defects as "Primary ciliary dyskinesia" and "Dysplasia of the fibrous sheath". The evaluation of cytoskeleton represents a specific and sensitive analysis in establishing the health status of different cells.

As a viewpoint, the manuscript describes personal considerations on the cytoskeleton, as the main outcomes of own research activity.

Cytoskeleton is an interconnected dynamic network of filamentous (comprising microtubules, microfilaments, and intermediate filaments) and regulatory proteins involved in controlling cell shape, cargo transport, signal transduction, and cell division. Both internal and external signals can act through the cytoskeleton to affect cellular behavior (Fletcher and Mullins, 2010).

The cytoskeleton, due to the interconnections with organelles and plasma membrane, is critical in mediating physiological, adaptive and degenerative cellular processes.

Characteristically, the analysis of cytoskeletal structure, in its component and dynamic variations, is useful to evaluate cellular state in a variety of conditions. Microscopic evaluation appears to be one of the most appropriate techniques to estimate cytoskeletal network, in addition to molecular or biochemical approaches applied to investigate single cytoskeletal protein components.

One of the functional peculiarities of the cytoskeleton is its dynamism, which provides structural support to cellular functions. The microenvironment strongly influences the cytoskeleton behavior: By a chain reaction, changes in the microenvironment are able to affect cytoskeleton assessment, which in turn can lead to severe pathological effects. If these alterations involve germ cells, hereditary consequences can be possible, although the triggering event is a reversible alteration. Thus, in addition to the evaluation, even quantitative, of the distinct proteins, the study of the cytoskeleton organization and its dynamism is relevant for the assessment of possible cellular pathological conditions such as neurodegeneration, cancer, infertility and others.

In line with these evidences, promising drugs, targeting the cytoskeleton, are currently tested in animal models, in human clinical trials and in *in vitro* studies (Hall, 2009; Kounakis and Tavernarakis, 2019).

Immunofluorescence (Geminiani *et al.*, 2017) and transmission electron microscopy are both valid methods to study integrity and distribution of cytoskeletal filaments (Pascarelli *et al.*, 2015) in physiological and pathological conditions.

The protein structure of the components and their interconnection makes the cytoskeleton particularly sensitive to oxidative damage, which then underlies many different diseases with common manifestations such as apoptosis and necrosis. Our research has often evaluated the injury of the cytoskeletal structure caused by oxidative damage induced by different etiological agents. Overall, taking into account our laboratory experiences, we observed that the lipid oxidative damage of cell membranes, detected by the formation of high malondialdehyde or isoprostanes levels, could be concomitant with protein (cytoskeleton) and DNA damage.

Over the years, our group was focused on the study of cytoskeleton in various cellular models and pathologies.

For example, structural differences at the nuclear, cytoplasmic, and cytoskeletal levels (Figs. 1A–1D) were observed (Pascarelli *et al.*, 2015) between cultured normal

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Received: 25 June 2021; Accepted: 13 August 2021

Doi: 10.32604/biocell.2022.018062

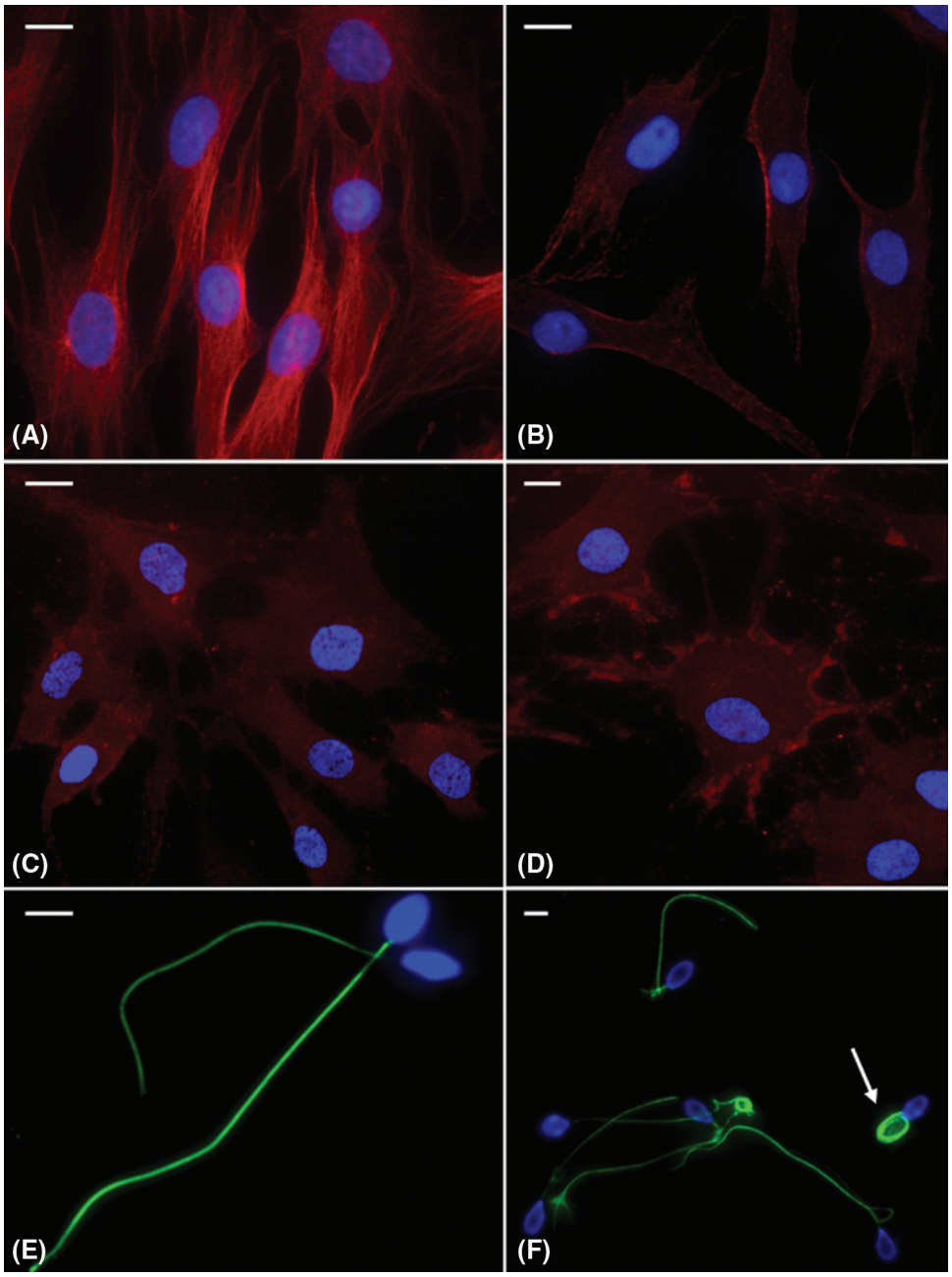

FIGURE 1. Indirect immunofluorescence microscopy.
(A) Normal chondrocytes incubated with monoclonal anti β-tubulin antibody revealed by a goat anti mouse antibody-rhodamine conjugated show a signal diffused from the nucleus to the periphery of the cytoplasm; after incubation with IL-1β a reduction of the fluorescence and disassembled tubulin filaments are evident (B). In (C) and (D) normal and altered chondrocytes were incubated with monoclonal anti actin antibody. The change of the label was evident. In (E) and (F) human sperm treated with a monoclonal anti-tubulin antibody, revealed by a goat anti mouse antibody-FITC conjugated, display the label in the sperm tail. This method enables to discriminate normal tails (E) and tails with multiple alterations including coiled tail (F, arrow). Nuclei (blue) were stained with DAPI. Bars: A, B, E, F) 50 μm, C, D) 5 μm.

chondrocytes and chondrocytes from patients with osteoarthritis (OA), a pathology that is a major source of pain, disability, and socioeconomic cost worldwide (Glyn-Jones *et al.*, 2015).

In both normal and OA chondrocytes, distribution of actin and tubulin appeared to be sensitive to changes in hydrostatic pressure in turn influencing collagen and proteoglycan synthesis. On the contrary, a cyclical low hydrostatic pressure counteracted the negative action of increased levels of IL-1β maintaining a regular distribution of the cytoskeletal proteins as actin, vimentin, and vinculin (Pascarelli *et al.*, 2015).

Cytoskeletal alterations were present also in chondrocytes of patients affected by alkaptonuria (AKU), an ultra-rare autosomal genetic disorder caused by a defect in the activity of the enzyme homogentisate 1,2-dioxygenase. Although AKU is a multisystemic disease, the most affected tissue is the articular cartilage. In AKU chondrocytes, the co-localization of serum amyloid A protein within actin, vimentin, and β-tubulin was observed (Geminiani *et al.*, 2017).

Still in the field of genetic diseases, an altered cytoskeletal organization, evidenced by a beta-actin down-regulation and oxidative posttranslational modifications, has been observed

in erythrocytes (Cortelazzo *et al*., 2014) of patients with Rett syndrome, a developmental disorder with an X-linked dominant inheritance pattern, mainly caused by mutations in the methyl CpG binding protein 2 (MECP2) (OMIM #300005) gene. Alterations of erythrocyte cytoskeleton are characteristic of other neurological genetic diseases (de Franceschi *et al*., 2014).

In vitro studies have been instrumental in testing the efficacy of anticancer therapies demonstrating the alterations in cytoskeletal organization that leads the cell to apoptosis. Micheli *et al.* (2021) reported that neuroblastoma cancer cells (SH-SY5Y) exposed to docetaxel showed a compromised organization of tubulin associated with an increased number of cells with apoptotic characteristics.

The cytoskeletal changes play an important role in the extraordinarily complex and dynamic seminiferous epithelium. Sertoli cells interact among them mainly with actin-based junctions that form the blood-testis barrier, a non-static immunological structure that divides the basal compartment, where spermatogonia undergo mitosis, and adluminal meiotic compartment. Environmental toxicants, as cadmium and bisphenol A, play their initial actions at the cytoskeleton based blood-testis barrier inducing testicular injury (Cheng and Mruk, 2012). Germ cells rely on unique and complex cytoskeletal dynamics to facilitate their development from a round, diploid cell into a haploid, highly specialized spermatozoon (Dunleavy *et al*., 2019). Microtubule based structures such as manchette and axoneme (Fig. 1E) are crucial for sperm head and tail formation. Our experience in the study of spermatozoa suggests that, among cytoskeletal proteins, tubulin is the one most involved in changes and alterations of cytoskeleton. In particular, tubulin glycylation, almost exclusively found in cilia and flagella, controls male fertility (Gadadhar *et al*., 2021). Many mutations of genes encoding cytoskeletal proteins are responsible for male infertility and can cause conditions such as "Primary Ciliary Dyskinesia" characterized by abnormalities of axonemal structure in particular of dynein arms, radial spokes, abnormal number of microtubules. "Dysplasia of Fibrous Sheath" and "Multiple Morphological Abnormalities of the Sperm Flagella" (Fig. 1F) are both conditions characterized by severe alterations of cytoskeleton of sperm tail (Moretti *et al*., 2017; Gunes *et al*., 2020).

Altered environment (presence of inflammation, anatomical problems) during spermatogenesis may affect the sperm cytoskeletal structure causing infertility, reduced reproductive outcome and embryo quality.

Varicocele, a pathology with multifactorial etiology, negatively influences testis microenvironment and several mechanisms have been suggested to explain its pathophysiology (Alsaikhan *et al*., 2016). Alterations in sperm morphology and presence of sperm aneuploidies, since microtubules damages affect the mitotic spindle (Baccetti *et al*., 2006), are possible in presence of varicocele.

Cytoskeleton organization can also be affected by the presence of nanoparticles in the microenvironment. While the number of nanoparticle types and applications is constantly increasing, the studies of their toxicity are scant. Silver nanoparticles, administered intravenously on New Zealand White rabbit bucks, determined persistent inflammatory status damaging the junctions of the blood-testis barrier and the interactions Sertoli germ cells (Collodel *et al*., 2020).

Although the cytoskeleton appears to undergo alterations caused by cellular interaction with different/several types of etiological agents, evaluation of cytoskeleton remains a specific and sensitive analysis in identifying cellular insult and cellular response to biological stimulation/damage. For example, cytoskeletal alterations are useful for the interpretation of necrotic and apoptotic events.

Our own research has shown cytoskeletal impairment due to the action of physical (Pascarelli *et al*., 2015), chemical (Micheli *et al*., 2021; Collodel *et al*., 2020), and genetic causes (Moretti *et al*., 2017) of disease. Here, the relevance of cytoskeleton alterations, as a common feature in different pathological conditions, is underlined. In particular, from our point of view, i) the cytoskeleton can be studied for the evaluation of cellular damages in case of treatment with new anticancer compounds and nanoparticles to establish their eventual toxicity; ii) the cytoskeleton assumes particular relevance in the field of male infertility and assisted reproduction technologies, in that sperm motility alterations, chromosomal segregation, and systematic sperm defects reflect cytoskeletal alterations.

Authors' Contribution: The authors confirm contribution to the paper as follows: study conception and design: C. Signorini, G. Collodel, E. Moretti; data collection: G. Collodel, E. Moretti; analysis and interpretation of results: C. Signorini, G. Collodel, E. Moretti; draft manuscript preparation: C. Signorini, G. Collodel, E. Moretti. All authors approved the final version of the manuscript.

Ethics Approval: All patients provided written informed consent for the inclusion in Center's researches according to the guidelines of the period for respecting privacy and the Helsinki Declaration of 1975. The study protocol involving chondrocytes was approved by the Ethics Committee of the Azienda Ospedaliera Universitaria Senese, Siena, Italy (Decision No. 726/07).

Funding Statement: The authors received no specific funding for this study.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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