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Literature review on the case of Gyps vultures populations decline due to diclofenac toxicity

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Abstract

Starting from 1990, three species of vulture endemic to the Indian sub-continent faced a steep decline and are now (2024) still classified as critically endangered by the International Union for Conservation of Nature (IUCN). Studies at Keoladeo National Park, where the affected species were residents, reported a decline of 96% and 97% for White-Backed and Long-Billed vultures respectively in the interval time considered (1985-1999).

Preliminary hypotheses on the cause of such a decline were formulated but it was in 2004 that Oaks et al. linked the vulture deaths to the veterinary use of diclofenac, a non-steroidal anti-inflammatory drug (NSAID). In response to this crisis, the drug has been banned nationally for veterinary use starting from 2006. However, in some areas, due to the persistent illegal sale, the already critical conditions of the species did not improve as much as expected.

Given that vultures react poorly to many other NSAIDs, the identification of safe alternatives is a priority. Meloxicam is one of those that have been tested and proven to be safe.

1. Introduction

Biodiversity is fundamental for the processes that can sustain life, such as oxygen production, therefore a wide variety of animal, plant and microorganisms species is essential for the maintenance of healthy ecosystems.

Nowadays, an increasing number of species are facing the risk of extinction. The causes of this biodiversity loss are almost exclusively caused by human activities, such as poaching, persecution and habitat loss resulting from climate change or deforestation. Additionally, at times, species are involuntarily put at risk simply as a result of a lack of knowledge, like in the case described here which is about diclofenac unwanted effects on vulture species.

Luckily there are many conservation initiatives around the world working to protect endangered species and their habitats.

1.1 Vultures

The vulture family is formed by a total of 23 species that further divides into the old-world and new-world vultures. Old-world vultures are found in Europe, Africa, and Asia, while new-world vultures are found in North and South America [5].

Old-world vultures are characterized by featherless heads, which help them stay clean from bacteria and parasites while feeding, and their exceptionally acidic stomach with a recorded pH of 1.0 [7], allows them to safely feed on decomposing carcasses possibly infected by bacteria such as anthrax [8], brucellosis, and tuberculosis [8][21].

Often associated with death and decay, they play an important role in the ecosystem by maintaining its balance as they are the only terrestrial obligate scavengers [6], meaning that their diet consists exclusively of dead animals. Vultures are excellent in locating carrion, and by gathering in big groups at feeding sites they can consume a carcass in a matter of 15 to 20 minutes [71] thus controlling problematic facultative scavengers such as feral dogs and rats [22], decreasing the risk of transmission of diseases such as rabies of which sadly India holds 36% of world's deaths [23].

1.1.2 Importance of Indian vultures for the Parsee community

The Parsi or Parsee community, whose name means "Persians", are descendants of Persian Zoroastrians who moved to India to avoid religious persecution by Muslims [27]. Zoroastrianism, is a monotheistic religion, and as a fundamental belief it has the existence of two distinct entities,

one being Ahura Mazda, wise and benevolent, creator of the world and all the good things in it, and Angra Mainyu, the "destructive spirit", the antagonist of Ahura Mazda, responsible for all negative things in the world, with death being one of them.

The sky burial is among their customs and rituals. As death is believed to be the highest form of pollution, since it only exists because of Angra Mainyu, the body is neither buried nor cremated as it would pollute the sacred elements of fire, earth, and water. Therefore bodies are laid to rest on Towers of Silence i.e. roofed stone towers, and then vultures quickly come to consume them. [28]

With the disappearance of these animals, sky burials in India have become ineffective, making it necessary for the Parsee community to find other suitable burial methods [29].

At present, to dehydrate the body faster, solar concentrators have been introduced, however, during the monsoon season, they don't work because of the clouds; and as cities have grown larger, complaints have arisen due to decomposing bodies being on sight [10].

The latest solution adopted by the community to this problem is electric cremation [20], also known as "flameless cremation" which uses water and heat [9] rather than fire, the element symbol of God's purity [28], to break down the body. The need for these innovations with their limitations emphasize the value of simplicity of the original tradition where a healthy vulture population is indispensable for the community.

1.2 The case of Gyps vultures' mass decline due to diclofenac toxicity

The species affected the most by this mass decline are Oriental White-Backed Vulture (*Gyps Bengalensis*), Long-Billed Vulture (*Gyps indicus*) and Slender-Billed Vulture (*Gyps tenuirostris*) [1], three species part of the Gyps genus, also known as griffon vultures, are part of old-world vultures.

Their exceptionally high population numbers declined by more than 95% in a decade, going from 62 individuals per sq. Km before 1990 to 3 per sq. Km during 1998-99. [1], reaching numbers of 4000-6000 individuals for the White-backed vulture, 5000-15000 individuals for the Long-billed vulture, and 730-870 for the Slender-billed vulture [24][25][26]. So that all three species share the 'Critically Endangered' conservation status, also known as the red label in the IUCN list.

The first reports about mortalities were described by Prakash et. al. in a study of 1999 performed in Keoladeo National Park, India [1]. Even though most of the dead vultures' carcasses probably went unnoticed due to factors such as other animals feeding on them, the ones described were often found dead on the ground below the trees, inside their nest, or entangled in

branches. In cases in which the animals were not dead yet, severe states of depression were manifested as a peculiar droopiness of their neck [1] (Figure 1).

Figure 1: A sick long-billed vulture. Photo by Nikita Prakash [65].

1.3 Hypothesis for the decline

At the time of this study, the cause of the mortality was still unknown. Therefore many hypotheses which considered a variety of ecological factors were examined. Some of the first factors investigated were the lack of food supply and the decrease of nesting and perch sites, but they were quickly ruled out as the cattle carcasses were still largely available and with few to no vultures feeding on them; while the lack of perching or nesting sites was unlikely to have caused such a drastic decline.

Another hypothesis advanced was considering an adverse effect of Pesticides and Insecticides, since the use of organochlorine pesticides was extensive in India, and their negative effect on bird health such as eggshell thinning, embryo death and occasional adult mortality had already been recorded during the years [1][2]. Moreover, since vultures are obligate scavengers, they are subject to biomagnification, which is the process by which toxic chemicals build up within organisms that are at higher levels in food webs [64]. As a matter of fact, obligate scavengers are more prone to accumulate harmful chemicals that were not broken down when the eaten animal was still alive [63].

Some vultures showed all the symptoms of pesticide contamination ranging from breeding failure to nestling death to high adult mortality. But the way the animals could have ingested such high doses of these chemicals was unclear. As mainly feeding on mammals, a class that has a better metabolization of organochlorines compared to birds and fish, vultures should have been

less susceptible to adverse effects [1]. Said that, if pesticide contamination was severe, the first animals to show critical cases of mortality should have been fish-eater and bird-eater birds.

Consequently other hypotheses were considered. Firstly, genetic differences in the metabolization of these compounds could have been a possible explanation, but this hypothesis was quickly rejected after the analysis of a few vulture tissues from Keoladeo Park which did not show any significant load of pesticides, thus excluding the involvement of Organochlorines as the main factor in the Indian vulture decline [1].

Secondly, the outbreak of a disease was also examined, because, even though Vultures are generally more resistant to bacterial diseases [3] due to their lifestyle, there is still a possibility of contracting viral diseases [1].

This was one of the theories left that was not ruled out until 2004, when Oaks and co-workers demonstrated that diclofenac, a drug with widespread use in veterinary medicine, was the cause of the Vulture mortalities.

The research team noticed that most of the birds had visible urate deposits on the surface of multiple internal organs, characteristic of a disease called Visceral Gout (Figure 2), which is often caused by renal failure. To determine the renal disease, diagnostic tests were performed on a set of 42 Oriental white backed Vulture (OWBV), and 28 of them showed the presence of acute renal tubular necrosis with uric acid crystal formation. Molecular analyses detected diclofenac residue in the kidneys of 25 out of 25 (100%) vultures that died of renal failure, and in 0 out of 13 (0%) vultures that died from other causes [4].

Further testing to verify the renal toxicity of diclofenac were conducted with a set of non-releasable captive individuals of OWBVs. Three out of four individuals died 36-58 hours after diclofenac administration, portraying the same microscopic renal lesions as the other set of wild individuals mentioned before [4].

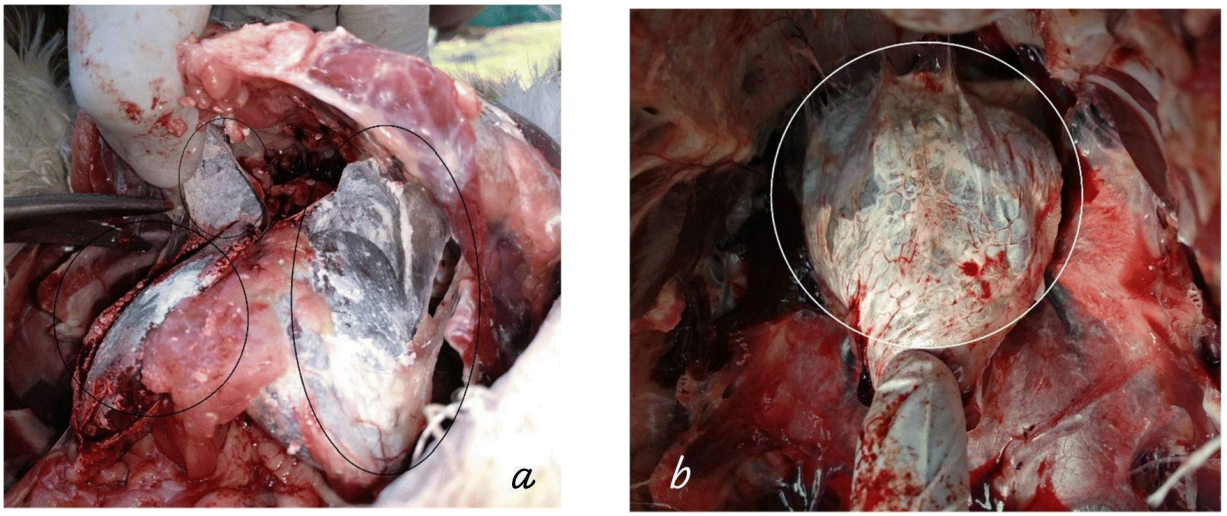

Figure 2: White chalky uric acid deposit on both the liver lobes (a) and in pericardium of heart (b) of a white-rumped vulture collected from Sanand, Gujarat [62].

2. Diclofenac

Diclofenac is a NSAID with COX-2 selective inhibitory activity [67], commonly used in human medicine for its analgesic effects to manage problems related to the musculoskeletal system, such as back pain or osteoarthritis. Additionally, it can also be used to manage conditions like gout or migraines [66]. From a veterinary perspective, diclofenac is primarily administered to alleviate pain during calving or provide relief from lameness, mastitis, and swelling [68].

2.1 NSAIDs

Non-steroidal anti-inflammatory drugs (NSAIDs) are often used to alleviate pain, or symptoms of inflammation and fever. This class of pharmaceuticals, by coming in a lot of different formulations from tablets to gels and injections, is one of the most used drugs in human and veterinary medicine [30] .

NSAIDs act on Cyclooxygenases, mainly COX-1 and COX-2, classes of enzymes that modulate certain types of physiological mechanisms.

COX-1 has important roles in gastrointestinal mucosa protection, platelet aggregation, and renal function, while COX-2, an enzyme that is upregulated in response to inflammatory and other physiologic stimuli, is involved in the production of those prostaglandins that mediate pain and support the inflammatory process [31].

The chronic use of older NSAIDs like aspirin and ibuprofen can cause serious gastrointestinal side effects by blocking both COX-1 and COX-2 enzymes [32][33]. To address this issue, companies have developed COX2-selective NSAIDs that mainly inhibit COX-2, reducing the risk of stomach ulcers and renal issues compared to non-selective NSAIDs [34].

2.2 Exposure to Diclofenac

With studies confirming the presence of Diclofenac as the cause of urate deposits on the surface of internal organs of 28 out of 42 adult and subadult OWBVs [4], it has been concluded that the exposure came directly from the Vulture's food source, in their case bovine carrion that was treated previously with the NSAID [35].

To understand how Vultures were exposed to diclofenac on such a large scale, it is essential to consider the religious and cultural customs of the countries involved predominantly in the decline: India, Nepal, Pakistan and Bangladesh.

Hinduism is a global religion originating in the Indian subcontinent and is the main belief of 80% of the Indian and Nepali population [36]. Hindu people hold the cow as a sacred animal, which represents divine and natural beneficence and should therefore be protected and venerated [37]. Accordingly they will not farm cattle for their meat, on the contrary, the common use of diclofenac was the effort of the farmers and the vets to give the animal a chance to heal rather than being slaughtered. In the case the animal died, a common practice among Indian farmers was to establish 'Vulture restaurants' [38], where carcasses were left intact to provide food for the once abundant Vulture population [39].

The same practice was also performed by Pakistani and Bangladeshis people. Pakistan and Bangladesh have more than 90% of the population practicing Islam [4]. Here cattle were held for meat production and farmers were administering sick animals with diclofenac because, according to the Islamic law, if an animal can be cared for and treated, it shall not be killed [69]. Additionally even if after the treatment the animal dies, its consumption is considered forbidden or 'haram' because its death was not caused by proper slaughter [41]. As a result, the diclofenac-treated animals were, once again, discarded away to be consumed by vultures.

2.3 Potential mechanisms leading to toxicity

Avian kidneys differ from the mammalian over many aspects, their most peculiar characteristic is the presence of two types of nephrons, one with and one without a loop of Henle [42].

While the mammalian kidney gets supplied by blood from the renal arteries splitting into arterioles [43], in the avian kidney the renal artery furnishes the glomerular part, while the peritubular areas are irrigated both by efferent glomerular arterioles and venous return from the legs which enters the external iliac vein communicating with the Renal Portal System (RPS). RPS is an additional blood supply (other than the arterial one) thought to provide half to two-thirds of the blood to the kidney.

The magnitude of blood from this system appears to be controlled by a valve called renal portal valve [44]. The renal portal valve is an autonomically innervated smooth muscle valve found within the renal portal circulation and can be located at various positions in the veins and is thought to be activated during stress conditions [4].

2.3.1 Renal Ischemia

With post-mortem findings of diffuse visceral gout with evident uric acid deposits, it has been proposed that the kidneys or their supportive vascular system are the site of toxicity [4].

Theories have been formulated to explain the toxic mechanism that killed millions of vultures. One of the hypotheses proposed by Meteyer et al., suggested that nephrotoxicity was caused by the diclofenac's COX inhibitory activity. This inhibition reduced the production of renal prostaglandins, leading to the closure of renal portal valves. Consequently, severe renal ischemia occurred, resulting in cellular damage and decreased uric acid excretion [46].

This hypothesis, despite being correct about COX inhibitory activity on prostaglandin formation, it seems to lack accuracy under other anatomical and physiological aspects of the avian kidney and renal portal valve [47]. Contrary to what Meteyer suggested, the valve needs to be opened to deprive the kidney of its blood supply; furthermore, the valve control is mediated by the beta-adrenergic and muscarinic receptors and not prostaglandins. The release of adrenaline, in response to stress conditions, leads to valve dilation, while acetylcholine promotes valve closure during normal physiological functioning [45].

Nevertheless, the avian kidney being characterized by a dual blood supply [44], makes the theory of complete tubular ischemia unlikely solely by the conditioned functioning of the valve, since as previously mentioned, the organ is additionally supplied by the renal artery and the portal vein.

2.3.2 OAT channel inhibition

Another theory advanced is on how diclofenac could interfere with the Uric Acid Excretion mechanism [47].

The kidneys play an important role in maintaining homeostasis by eliminating uric acid from the body, a process that involves glomerular filtration and tubular reabsorption and secretion [48].

The glomerular filtration process is possible due to differential pressure across the glomerulus in a size-dependent manner, while the management of uric acid in the proximal convoluted tubules is mediated by a variety of proteins that differentiate into transport proteins (OAT1 to OAT4), multidrug resistance proteins (MRP) and urate transporter 1 (URAT1) [49].

In mammals, OAT1 and 3 are localized in the basolateral membrane of proximal tubules and are responsible for the transport of Uric Acid from the blood vessel into the intracellular environment. From here MRP transporters have the function of excreting Uric Acid from within the cytoplasm into the renal tubule lumen. After uric acid gets filtered by the glomerular and tubular systems, if it's not directly excreted with urine, it can be reabsorbed by URAT1 in the process named tubular reabsorption [50].

Vultures and other birds have a higher excretory demand for uric acid and the glomerular filtration is not enough. As a result, their tubules are responsible for up to 80% of the total excretion of uric acid [51]. To further enhance its excretion, the reabsorption does not occur, explaining why URAT1 transporters in birds have not been described yet [52].

Diclofenac is thought to inhibit uric acid transport inside the cell by interfering with OAT channels. This deprives the cell of an antioxidant while, at the same time, it is being exposed to greater production of Reactive Oxygen Species (ROS) [47] resulting from the oxidation of NADPH by O2, caused by the continuous conversion of 4′-hydroxydiclofenac into 5′-hydroxydiclofenac major and minor metabolites of diclofenac formed after its bioactivation during phase 1 metabolism [70]. This leads to oxidation of the mitochondrial membrane proteins. As a result of this damage, the mitochondrial membrane becomes permeable, allowing the release of pro-apoptotic factors such as cytochrome-c into the cytoplasm. Cytochrome-c then activates caspases, initiating the apoptotic process. [54].

Moreover the absence of glycosylation sites for OAT1, which was present for OAT2 suggests that OAT1 may not be a functional transporter. In fact these proteins are usually located within intracellular vesicles and need to be moved to the cell membrane to become functional, which is

unlikely in the absence of glycosylation sites, which play an important role in the attachment of the protein onto the plasma membrane [52].

3. Mitigation strategies

3.1 Safe alternatives

Vultures experiencing toxicity not just to diclofenac but also several other NSAIDs available in the market made the need for a safe alternative a priority.

Following a study in 2006, meloxicam was labeled as safe for India's critically endangered vultures and many other scavenging species. This drug was tested on 51 scavenging species including 31 individuals of Gyps Vultures, all the animals survived the study without experiencing any adverse effects [55].

Already approved for veterinary use in India, Europe, North America [29], Australia, New Zealand, the UK, and Ireland [56], meloxicam, commonly used as analgesic to treat arthritis and joint stiffness, was one of the first safe alternatives to diclofenac [57]. It was therefore promoted to pharmacists and farmers by conservationists across South Asia [58].

However, many of its injectable formulations were not preferred by the users because they caused pain to the animal during administration. This was due to the high pH and high osmolarity of India’s formulations [59], but after the patent for the original formulation that was used in Europe was released, Indian pharmaceutical companies began to replicate it [58].

Undercover pharmacy surveys performed in the Indian subcontinent, especially in Nepal and Bangladesh revealed that after meloxicam, nimesulide, and ketoprofen, another drug was increasingly offered for veterinary use, Tolfenamic acid [58].

Tolfenamic acid is an NSAID approved in the UK, some countries in Europe, Latin America, and Asia for both human and veterinary use [60] and was just recently marked as safe for vultures in 2021, following a study conducted by Chandramohan et. al. where they tested the drug on 42 vultures, with the results reporting that 40 birds survived, while 2 of them died [61]. Despite the deaths, the drug was considered safe because of the factors that may have contributed to the death of the two animals, these being the high dosage level used in the experiment, which are unlikely to be found in the wild, and the high ambient temperatures [61].

The discovery of this drug being safe is just another step further in finding many other safe alternatives, to give veterinarians and livestock owners more choices.

3.2 Diclofenac ban

After the research publication of Oaks et. al. in 2004 where he addressed the use of diclofenac in veterinary practices as the cause of the crash in vulture populations [4], the governments of India, Pakistan, and Nepal, banned the manufacture of veterinary formulations of the drug in 2006, followed by Bangladesh in 2010 [11].

Unfortunately, in India people continued to use diclofenac illegally, probably incentivized by the fact that Indian Pharmaceutical companies were producing human formulations in vials containing enough drug to dose cattle. This led to a strengthening of the regulations in 2015, which resulted in the ban on the production of vials bigger than a human dose [11].

Despite the initial ban on diclofenac, meloxicam was not easily available in India and Bangladesh, and it was often outsold by other NSAIDs such as ketoprofen and carprofen, which are toxic to vultures [12], and aceclofenac, which metabolizes into diclofenac in cattle [13].

Meloxicam was therefore promoted by national and local government bodies, by doing so they helped the drug to maintain its market share. These promotion campaigns were especially effective in Nepal, where surveys showed meloxicam as the most frequently sold drug [11].

4. Current situation

4.1 Safe drug availability and new bans

Over the years countries put efforts into promoting the use of meloxicam and Tolfenamic acid, by raising awareness among the population, in particular vendors, veterinarians, and of course, cattle owners.

A great number of pharmacy surveys have also been conducted to help understand trends in the availability of safe and harmful alternatives, and to take appropriate action based on the findings.

4.1.1 Pakistan

Recent Pharmacy Surveys in Pakistan, to assess NSAID availability and the vendor’s knowledge of the bans, reported no veterinary diclofenac on sale. However, meloxicam was available in all surveyed pharmacies but aceclofenac, ketoprofen, and flunixin were also present in more than half of them. Interestingly, while more than 90% of the vendors were aware of the diclofenac ban, 43% of them did not know the reason behind the ban [14].

4.1.2 Nepal

Since the ban on diclofenac in 2006, Nepal has made significant progress. In 2010 the first district was declared diclofenac-free, and now all 77 districts, consisting of the whole country of Nepal, are veterinary diclofenac-free.

The only significant threat left to Gyps vultures is accidental poisoning, thus becoming a new topic for awareness programs. Livestock owners, to protect their animals, often poison jackals and feral dogs, but this practice can also harm vultures who feed on the carcasses and die as a result; in one of these incidents, 9 individuals of White-backed vulture and 1 Egyptian Vulture were found dead [15].

4.1.3 India

A vulture release zone, in the state of Assam, which additionally contains a Vulture Safe Zone (VSZ), was surveyed in 2024 and revealed meloxicam as the most popular veterinary NSAID available. Tolfenamic acid was also becoming increasingly popular, however, it is still less common than nimesulide and aceclofenac, which were both declared to be toxic to vultures [18][13].

Fortunately, the availability trends of aceclofenac will change soon, as since August 2023 the Indian Government has implemented the international ban on both ketoprofen and aceclofenac [16].

Moreover, the Drugs Technical Advisory Board of India (DTABI) i.e. the highest decision-making body on matters regarding drugs in India, has recently recommended a national ban on the veterinary drug nimesulide. The Indian Health Ministry and Central Drugs Standards Control Organization (CDSCO) made this announcement following a meeting held in late January 2024 [19].

4.1.4 Bangladesh

In Bangladesh, the ban of ketoprofen in 2021 is incentivizing the sales of meloxicam and Tolfenamic acid accounting for 15,76% and 26.6% respectively.

According to the ongoing Undercover Pharmacy survey (2024), the availability and demand of Tolfenamic acid, and meloxicam exceeds the demand for flunixin, which even if it is present in more than half of the pharmacies surveyed, its sale is limited due to its high price [17].

4.2 Conservation efforts

In countries with the most depleted vulture populations, significant efforts are in progress to protect and improve their conservation status. Here are some examples:

- Captive breeding centers were established in Pakistan, India and Nepal, in order to maintain viable populations in captivity, with the eventual goal of producing vultures for release back into the wild [14]. Nepal has been successful in its attempt to release captive individuals. Out of 12 captive vultures seen in breeding behavior, 7 were able to successfully fledge their young in the wild. Additionally, Nepal recorded a 71% breeding success rate in wild vulture populations, which has been improving each year, this success is likely due to the effective addressing of the threat of toxic NSAIDs [15].
- Vulture Safe Zones (VSZ) play a crucial role in safeguarding vulture populations, and all the countries have some established [14][15][16][17]. In these special areas, extra efforts are made to raise awareness through programs and meetings for veterinary personnel, pharmacy vendors, and livestock owners, to promote a deeper understanding of the critical situation of Vultures and, therefore, to understand the importance of VSZ.

  These areas are not just ideal for keeping a close eye on population trends thanks to road transects, tagging, and radio location, but they are also the best locations to release rescued or rehabilitated birds.
- Pharmacy surveys were performed intensively in all the countries listed before, particularly in pharmacies inside VSZ to stay up to date with drug availability trends.
- Provision of safe food in areas known to be inhabited by wild populations of vultures.
- Awareness initiatives offered to all parties involved with NSAIDs, whether they produce, prescribe, sell, or purchase them [14][15][16][17].

5. Conclusion

All the efforts put in until now since the first ban of Diclofenac in 2006, have shown to be slowing, or at least stabilizing the decline in vulture populations in most of the countries, for instance road transects in India counted 106 White-rumped Vultures, 299 Indian Vultures, and 11 Slender-billed Vultures in 2022, compared with 102, 139, and 12 in 2015.

However, Nepal has experienced a significant increase in numbers, starting with less than 50 active nests in 2006 it reached over 600 by 2023. Nepal's quick approach to address NSAIDs toxicity should be imitated, and more awareness campaigns and sensitization programs should be offered in all countries impacted.

The availability of toxic alternatives of NSAIDs is often higher than the one of meloxicam, and tolfenamic acid, which since it has been recently introduced is still relatively uncommon.

However, changes in these trends are expected, thanks to the recent bans on ketoprofen and aceclofenac.

Nimesulide is increasingly available despite being toxic to Vultures. The proposed ban in India gives hope for population growth, providing new possibilities to stiffen the regulations immediately, without delaying the situation from getting worse.

After spending the entire duration of the internship in a Rehabilitation and Rescue Center for birds of prey where a young Griffon Vulture was taken care of, my admiration for these creatures increased significantly. While vultures may not be as charismatic as other endangered species, their role in the ecosystem should be acknowledged, and by raising awareness and people's sympathy towards these animals, we can better contribute to their conservation.

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